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Organization of acetylcholine receptor (AchR) and acetylcholinesterase (AchE) in denerwated skeletal muscle of rat.

University — Université

University of Alberta

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Doctor of Philosophy (Ph.D.)

Year this degree conferred — Année d'obtention de ce grade

Spring 1980

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ORGANIZATION OF ACETYLCHOLINE RECEPTORS (AchR) AND ACETYLCHOLINESTERASE (AchE) IN DENERVATED SKELETAL MUSCLE OF RAT

by

ULKA R. TIPNIS

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

CELL BIOLOGY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SPRING, 1980

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Organization of acetylcholine receptors (AchR) and acetylcholinesterase (AchE) in denervated skeletal muscle of rat," submitted by Ulka R. Tipnis in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Cell Biology.

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ABSTRACT

The present investigation is directed towards identifying two macromolecular components of skeletal muscle of adult rat, namely (i) peripheral membrane protein acetylcholinesterase (AchE) and (ii) integral membrane protein acetylcholine receptor (AchR). Advantage is taken of the current biochemical and physiological understanding that these proteins undergo marked alterations following denervation of the muscle, that is while AchE decreases (Guth et al., 1964; Hall, 1973), AchR are synthesized and incorporated into the non-synaptic (extrajunctional) sarcolemma (Devreotes and Fambrough, 1975). This incorporation of AchR causes noticeable increase in the AchR in the extrajunctional sarcolemma accounting for the well known acetylcholine (Ach) sensitivity of this region in denervated muscle (Axelsson and Thesleff, 1959).

My studies related to AchE are based on a histochemical technique to localise specifically AchE, in the region of the neuromuscular junction (synapse) using an inhibitor (BW284C51) specific for AchE. An attempt has been made to understand the relationship, if any, of AchE molecules to the macromolecules identifiable as intramembranous particles in the hydrophobic interior of the membrane.

The results of my studies indicate that it is possibly the 16S molecular form of AchE that produces a uniform reaction product in the synaptic cleft. Upon denervation, this AchE undergoes a marked decrease that is characterised by its non-uniform distribution. In the denervated muscle, the structure of synaptic sarcolemma shows no obvious change in

the number of intramembranous particles visualised in freeze-fractured replicas. Based on these experiments it appears that the intramembranous particles are not related to the AchE molecules.

Acetylcholine receptors have been investigated in innervated (control) and denervated muscle by combination of freeze-fracturing and etching and autoradiographic technique using α-bungarotoxin (α-BGT), which is a specific ligand for AchR. The experimentation by freeze-etching technique involves the use of a ferritin-α-BGT (Ft-α-BGT) conjugate to visualise the receptors at the surface of the membrane. Autoradiographic experiments have involved the use of ^{125}I-α-BGT to study the distribution and quantitation of receptors.

My previous studies (Tipnis and Malhotra, 1976) employing freeze-fracturing technique indicated that the non-synaptic sarcolemma of denervated muscle is characterised by 15 nm (15-18 nm) intramembranous particles distributed singly or in clusters on the P-face. The corresponding fractured face of the innervated muscle is devoid of 15 nm particles. On the basis of published physiological and biochemical data (Hartzell and Fambrough, 1972; Brockes et al., 1975), it was suggested that the 15 nm particles are the components of AchR complexes. The results of the present study have provided support for the above suggestion. Both light and electron microscope autoradiography, by use of ^{125}I-α-BGT, indicate that there is an increase in α-BGT binding sites in the non-synaptic sarcolemma of denervated muscle. Two spatial distributions of AchR are noted: randomly distributed AchR and AchR in aggregates. This observation is in agreement with the freeze-fracturing data given above. The

quantification of ^{125}I-α-BGT binding sites by electron microscope autoradiographic studies indicates that the non-synaptic sarcolemma of denervated muscle has approximately 1,590 α-BGT binding sites/μm^2. This estimate is based on the assumption that the ratio of binding of α-BGT to receptor is 1:1. The density of 15 nm particles (AchR) noted in freeze-fracture studies is 400 particles/μm^2. The discrepancy in α-BGT binding sites and the density of 15 nm particles is possibly due to two or more α-BGT binding sites per receptor molecule (as suggested by Karlin et al., 1978).

Further confirmation of this suggestion has been provided by experiments which have utilised Ft-α-BGT in conjunction with freeze-etching and thin sections of crude sarcolemmal preparations or muscle homogenate prepared from denervated muscle. The results of this study reaffirm the two types of distributions of receptors in the non-synaptic sarcolemma of denervated muscle. The singly dispersed receptors correspond to the mobile AchR of Axelrod et al. (1976) and the clusters of AchR correspond to the hot spots of Ko and coworkers (1977).

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ACKNOWLEDGEMENTS

I am deeply indebted to Dr. S. K. Malhotra for his encouragement, guidance, assistance and patience in the completion of the project. He has shown extreme consideration towards my difficulties and have always expressed confidence and interest in the present study.

I wish to thank Dr. A. van Harreveld, Dr. G. Monckton, Dr. D. M. Ross Dr. N. R. Thomas and Dr. J. S. Nelson for critically reading the manuscripts. The encouraging words and the suggestions from the members of my supervisory committee have been helpful in the conduction of the present study.

The enjoyment in undertaking these studies would not have been complete without the pride and encouragement expressed by my parents and parents-in-law. I deeply appreciate Ravin for supporting wholeheartedly my own academic interest which has demanded a considerable degree of sacrifice and understanding from him.

I am extremely thankful to my friends, Mrs. Medha Karnik, Mr. and Mrs. Fell, Drs. Ranjana and Arun Nagpurkar, Dr. Suman Sahai, Santosh Sikerwar, and Dr. Tessa Gordon, for their valuable assistance.

I am grateful to Dr. J. P. Tewari for rendering valuable advice, assistance and cooperation throughout the course of this study. I wish to thank Mr. Steve Ross and Ms. Ann Boran for their technical help.

I am grateful to the Department of Zoology for providing financial assistance. Various persons of this department have always

expressed understanding and offered their assistance. I therefore would like to thank Ms. Mag Haag, Mrs. Carine Miner, Mr. William Robinson, Mr. Jerry Hadyma and Mr. Joe Bouvier. I am thankful to Helena Marusyk for the discussion on the technical details of autoradiography.

The research work submitted in this thesis has been either published or accepted for publication and the manuscripts were submitted under joint authorship with my supervisor.

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ABBREVIATIONS

The following abbreviations have been used in this thesis:

Acetylcholine	=	Ach
Acetylcholinesterase	=	AchE
Angstrom	=	Å
α-Bungarotoxin	=	α-BGT or BGT
β-Bungarotoxin	=	β-BGT
Cholinesterase	=	ChE
Difluorophosphate	=	DFP
Ferritin	=	Ft
Hour	=	h
Micron	=	μ
Nanometer	=	nm
Tetrodotoxin	=	TTX

CHAPTER I

General Introduction

INTRODUCTION

Biological membranes play a vital role in a wide variety of cellular phenomena, but our current understanding of their functioning is far from complete. In recent years, diverse technological and experimental manipulations have been instrumental in the formulation of a general model embodied in the fluid mosaic model (Singer and Nicolson, 1972). According to this model, the bulk of the lipid is in the form of an interrupted bilayer and the proteins are either bound to the surface of the bilayer or intercalated to varying degrees into its hydrophobic interior. Depending upon the ease with which proteins can be removed from the bilayer, they are operationally described as peripheral (extrinsic) or integral (intrinsic). Peripheral proteins can be dissociated from the membrane by manipulation of ionic concentrations of medium. Integral proteins, on the other hand, require much more drastic treatment, such as organic solvents or detergents for removal from the membranes (Singer and Nicolson, 1972; Reviewed by Malhotra, 1979).

In this thesis I have directed my studies towards the macromolecular organization of sarcolemma of rat skeletal muscle (lumbricals and extensor digitorum longus) in relation to two specific proteins. One peripheral and one integral protein have been considered and these are acetylcholinesterase (AchE) and acetylcholine receptors (AchR). The rationale for using mammalian sarcolemma as a model system for my studies is based upon the following characteristics.

1. Use of Sarcolemma as a Model System

The sarcolemma bears several characteristics not readily observed

in many other membrane systems and these are placed in the following two major categories.

(i) Compartmentalisation. The sarcolemma is compartmentalised distinctly, morphologically and physiologically, into synaptic and non-synaptic zone. The synaptic zone is characterised by extensive infoldings of the sarcolemma which contains predominantly two proteins AchE and AchR (Salpeter, 1967, 1969; Porter et al., 1973; Fambrough, 1979). It is believed that acetylcholine (Ach) released from the nerve terminal binds to AchR initiating the conformational changes that result in the opening of AchR channels to Na^+ and K^+ (Changeux et al., 1976; Heidmann and Changeux, 1978). The increased permeability to these cations gives rise to end plate potentials which if of sufficient amplitude generate the action potential of the muscle membrane, acetylcholinesterase hydrolyses Ach thereby terminating its action (Aidley, 1971). Based on the binding of H3-difluorophosphate (H3-DFP) there are approximately 8600-12000 AchE molecules/μm^2 in the synaptic sarcolemma (Salpeter, 1969; Porter et al., 1973). According to the current understanding, AchE exists in three molecular forms, 4S, 10S and 16S in mammalian muscle. While 4S and 10S are distributed throughout the sarcolemma, it is the 16S form which is end-plate specific (Hall, 1973).

Physiological studies have revealed that the maximum sensitivity to Ach lies in the region of the synapse of innervated muscle (Axelsson and Thesleff, 1959; Muledi, 1960). Experiments dealing with quantitation of AchR have shown that the density of the receptor molecules is extremely high in the synaptic region. The estimates of AchR vary from 10,000 to 30,000 receptors/μm^2 in the synaptic sarcolemma of innervated muscle

(Fambrough and Hartzell, 1972; Porter et al., 1973; Fertuck and Salpeter, 1976). The variation in density of receptors appears to be due to the particular experimental details rather than variation within mammalian muscle type. In contrast to the synaptic sarcolemma, the non-synaptic sarcolemma has a few receptors (approximately 5-10 receptors/μm^2) (Hartzell and Fambrough, 1972; Fambrough, 1979). These estimates of AchR are based on the binding of radiolabelled preparations of α-bungarotoxin (α-BGT) which is a specific and irreversible ligand for nicotinic AchR (Lee, 1979).

The above-noted biochemical and physiological experimental data are therefore conducive towards carrying out my own structural studies on the sarcolemma.

(ii) Denervation. Various biochemical and physiological experiments indicate that denervation produces alterations in the sarcolemma of mammalian muscle manifested in extrajunctional (also referred as non-synaptic) sensitivity to Ach (Axelsson and Thesleff, 1959; Miledi, 1960; Hartzell and Fambrough, 1972), and a decrease in AchE (Guth et al., 1964; McCaman, 1966; Hall, 1973). The increase in extrajunctional sensitivity to Ach results from the incorporation of newly synthesized AchR in the non-synaptic sarcolemma. Owing to these known effects of denervation on AchE and AchR, morphological characterization of these proteins could be accomplished and alterations in the structure of the sarcolemma investigated.

2. Studies on Acetylcholinesterase (AchE)

Aspect of this study pertains to the distribution of AchE in innervated and denervated muscle by a histochemical technique (Karnovsky and Roots, 1964) in which a specific inhibitor of AchE, i.e., 1,5-bis(4 allyldimethyl ammonium phenyl)pentane-3-one dibromide (BW284C51) has been employed. Although my previous studies (Tipnis and Malhotra, 1977) presented findings on the distribution of AchE, these were not conducted with the specific inhibitor of AchE, thus leaving the specificity of histochemical localisation open to question. The present study attempts to clarify this question. Therefore the objectives of this study in relation to AchE are the following:

(a) To confirm the specificity of the histochemical localisation of AchE by using BW284C51 as a specific inhibitor of the enzyme. This has been done at the level of electron microscopy.

(b) To seek the relationship between the molecular form of AchE and the identifiable macromolecule if any by correlative histochemical and freeze-fracture studies.

These two studies have been carried out on innervated and denervated lumbrical muscles of the rat.

3. Studies on Acetylcholine Receptor (AchR)

(i) Freeze-fracturing and etching technique. The rationale for the present investigation is based on the available biochemical and physiological data that are related to the increase in extrajunctional sensitivity to Ach in denervated muscle (Miledi, 1960; Edwards, 1979; Fambrough, 1979). The use of freeze-fracturing and freeze-etching

techniques is related to the fact that AchR is an integral protein (glycoprotein) of the membrane (Changeux et al., 1976; Fambrough, 1979) and projects outside and inside the bilayer by 5.5 nm and 1.5 nm respectively (Klymkowsky and Stroud, 1979). This receptor protein can be specifically labelled with α-BGT which upon conjugation to ferritin enables the visualisation of receptors at the surface of the membrane by freeze-etching.

In freeze-fracturing, biological membranes split the membrane bilayer into two halves. These membrane halves allow us to view the interior of the membrane revealing both the inner protoplasmic half (P or PF) and the outer extracellular half (E or EF). In freeze-etching, the ice is allowed to sublime in vacuum so that the membrane surface (ES) is exposed (Branton et al., 1975). These techniques therefore enable us to study both the macromolecular entities within the membrane interior as well as at the surface.

In denervated lumbrical skeletal muscle of the rat, I have previously demonstrated that the non-synaptic sarcolemma changes markedly after denervation (Tipnis & Malhotra, 1976). This change is related to the emergence of 15 nm (15-18 nm) intramembranous particles on the P face. There are two spatial distributions of these particles: single and in clusters of 5-30 particles. Such particles are conspicuous by their absence from the corresponding face of innervated muscle. On the basis of available biochemical and physiological evidence, it was suggested that the 15 nm particles are the components of AchR. The objective of the present project therefore is to investigate the above hypothesis and to demonstrate that the spatial distribution of 15 nm particles

corresponds to the distribution of AchR. Such an investigation is feasible by usage of conjugates of α-BGT with ^{125}I or ferritin (Ft) (Appendix II).

(ii) ^{125}I-α-BGT. The use of ^{125}I-α-BGT has been directed towards:

(a) The visualisation of the distribution of receptors by light and electron microscope autoradiography.

(b) The study of specific binding of α-BGT in muscles as well as crude membrane preparations (as detailed in Appendix III).

(c) Quantitation of the α-BGT binding sites and their correlation with the density of 15 nm particles observed on the P-face of non-specific sarcolemma (Appendix IV).

(iii) Ferritin-α-bungarotoxin (Ft-α-BGT). Ferritin is conjugated to α-BGT according to the method given by Hourani et al. (1974). Ferritin is an electron dense molecule (~10 nm) (Williams, 1977) and can be easily detected in electron micrographs. The conjugate (Ft-α-BGT) has been utilised for the following experimental approaches:

(a) The visualisation of α-BGT binding sites (AchR) in thin sections of crude sarcolemmal preparations.

(b) The correlation of the distribution of receptor sites by freeze-etching with that of intramembranous particles (~15 nm) observed by freeze-fracturing in non-synaptic sarcolemma of denervated skeletal muscle (Tipnis and Malhotra, 1976). Freeze-etching is feasible only in membrane fractions or homogenate as high molecular weight substances *in situ* do not sublime easily and the surfaces of the membrane cannot be visualised.

To sum up then, the specific objectives of this investigation are to characterise morphologically AchR and AchE of the sarcolemma in the skeletal muscle of the rat. I have attempted through experiments to either confirm or refine some previously published work (Tipnis and Malhotra, 1977). However, the important aspect of my work concerns the demonstration of two spatial distributions of intramembranous particles corresponding to two categories of AchR in the sarcolemma of denervated skeletal muscle of rat (*Rattus rattus*). This has been achieved by using ^{125}I-α-BGT or ferritin-α-BGT as a specific ligand for AchR in conjunction with current techniques of light and electron microscopy, namely, autoradiography, freeze-fracturing, etching and conventional transmission electron microscopy. The other main aspect of my work pertains to specific histochemical localisation of the 16S form of AchE in the synaptic cleft in innervated muscle which undergoes a marked decrease following denervation.

The investigative aspects of the thesis are organized in the following chapters:

Chapter II. Alterations in the structure of sarcolemma in a denervated skeletal muscle. This investigation deals with the study of the distribution of AchE in innervated and denervated skeletal muscle. An attempt is made to ascertain if there is a relationship between the AchE molecules (16S) and the intramembranous particles observed on the fractured faces of the synaptic sarcolemma.

Chapter III. α-bungarotoxin binding sites (acetylcholine receptors) in denervated mammalian skeletal muscle. This aspect of the project was undertaken to provide evidence to support the suggestion that

the 15 nm particles observed on the fractured face of non-synaptic sarcolemma of denervated muscle are components of the AchR. This objective has been achieved by correlating the distribution of 15 nm particles with the distribution of α-BGT binding sites by light microscope autoradiography (^{125}I-α-BGT) and by freeze-etching using Ft-α-BGT.

Chapter IV. Autoradiographic studies of α-bungarotoxin sites (acetylcholine receptors) in innervated and denervated muscle of the rat. This investigation deals with a qualitative correlation of spatial distribution of 15 nm particles (on the fractured face of the non-synaptic sarcolemma of the denervated muscle) with ^{125}I-α-BGT binding sites by light and electron microscope autoradiography. A qualitative correlation of the density of 15 nm intramembranous particles with that of α-BGT binding sites has been determined by electron microscope autoradiography.

Chapter V. Junctional and extrajunctional acetylcholine receptors. This chapter is a review which is intended to describe the distribution of the nicotinic AchR and the differences between the junctional and extrajunctional receptors. Included here is the discussion on the biochemical, physiological and pharmacological properties of AchR.

Chapter VI. Conclusions. This chapter deals with my conclusions arrived at from the experimental data in support of the investigation aimed at the objectives outlines in the introduction to this thesis.

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Volume 69, number 1 FEBS LETTERS October 1976

DENERVATION OF SKELETAL MUSCLE: CHANGES IN THE STRUCTURE OF NON-SYNAPTIC SARCOLEMMA

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Received 2 August 1976

1. Introduction

It is well known that in adult skeletal muscle acetylcholine (Ach) receptors are localized in the subsynaptic sarcolemma [4,5]. Denervation of muscle results in appearance of Ach receptors over the entire surface of the sarcolemma [6,7]. This increase of Ach receptors is due to incorporation of newly synthesized receptor proteins into the non-synaptic sarcolemma [2]. The biochemical and electron microscopic studies on receptor rich membranes isolated from electric organ of fish such as *Electrophorus electricus* and *Torpedo marmorata* indicate that the receptor protein spans the entire thickness of the membrane and is exposed at its surface [3]. Therefore, it is of interest to investigate the structural aspects of denervated sarcolemma at a stage when extra-junctional Ach sensitivity is known to be high [6], by using freeze-fracturing technique which enables a direct visualization of the interior of membranes [1].

This paper deals with our observations on the freeze-fractured sarcolemma from denervated skeletal muscle.

2. Material and methods

Lumbrical muscles of the left hind leg of the female rats weighing 120–150 g were denervated by sectioning the sciatic nerve in the upper thigh region. The muscles of the right leg served as controls. The animals were sacrificed at 2 weeks after denervation and the excised muscles were fixed in 2% glutaraldehyde in 0.1 M Cacodylate buffer (pH 7.2). The fixation was carried at 4°C for 2 h. After a brief rinse in cacodylate buffer, the muscles were soaked in 30% glycerol for 2 h. Small pieces of the muscles were then frozen in liquid Freon 22. Pt-c replicas were made after fracturing at −100°C in a Balzers BA 360 M high vaccum freeze-etch unit. The replicas were cleaned overnight in chromic acid and examined in Philips 300 electron microscope at an accelerating voltage of 60 or 80 kV. The original pictures were magnified to × 80 000 for measurement of particles. The results reported in this paper are based on a study of at least 12 replicas prepared from 12 muscles taken out of 3 denervated rats and many more replicas of normal muscles taken out from at least 10 animals.

3. Results

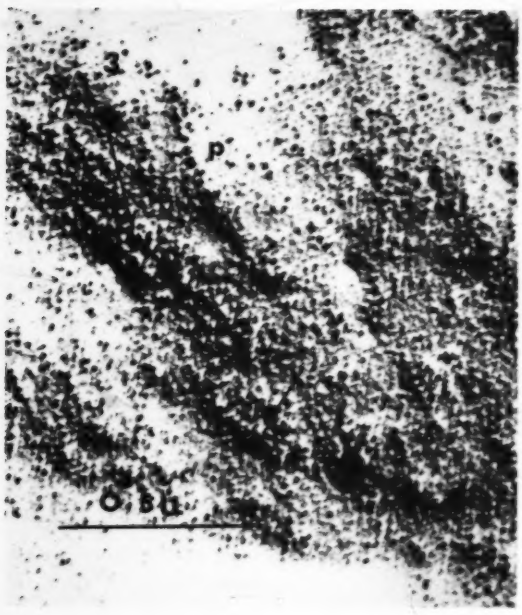
The nomenclature used by Branton et al. [1] for labelling fractured faces of biological membranes has been adapted in this paper. Therefore the fractured face of the sarcolemma on the cytoplasmic side as viewed from outside is labelled as face P and its complementary half is labelled E.

3.1. *Non-synaptic zone of innervated sarcolemma*

The fractured face P of the sarcolemma in control muscle is characterized by numerous particles which are mostly 80 Å in diameter (fig.1A). The particles are rather uniformly dispersed and their density is approximately 2000/μm^2. The orthogonal arrays similar to those described by [8] are also of common occurrence. There are also numerous depressions of 300–500 Å diameter which are presumably openings of cortical vesicles or T-tubules. Fractured face E shows numerous protuberances that are complemen tary to the 300–500 Å depressions on face P. This face shows far fewer 80 Å particles than on face P.

A

B



C

D

3.2. *Non-synaptic zone of denervated sarcolemma*

The replicas prepared from denervated muscle show marked changes in the structure of the fractured face P of the sarcolemma as compared to that on the corresponding face in the control muscle. Some of these fractured faces are conspicuous by the presence of aggregates (figs.1C,D) of particles which are mostly 110 Å to 180 Å in size. The number of particles in each aggregate varies from 4–50 or more particles. These aggregates are randomly dispersed over the P face; but the number of such aggregates varies in replicas. It is apparent that such aggregates of particles are not discerned on either of the fractured faces of the sarcolemma in the control muscle. Also the size of these particles is different from the particles in the control muscles. Moreover, the 80 Å particles that are predominant on the P face of the sarcolemma of the control muscle are reduced in number. Many fractured faces where aggregates are not observed, the particles are randomly distributed and have packing density of 400–1000/μm^2 (fig.1B) as compared to 2000/μm^2 in those of control.

The E face of the denervated sarcolemma is relatively unchanged, there being no groups of depressions complementary to the aggregates on the P face. Such an asymmetry may result from a preferential attachment of the particles to the cytoplasmic half of the membrane as has been described for intramembranous particles representing bacteriorhodopsin in the purple membranes of halobacteria [9].

4. Discussion

The above studies indicate that denervation induces the formation of aggregates of large particles. The size of many of these particles in the aggregates is consistent with that reported for Ach receptors by Rash and Ellisman [8] in mammalian neuromuscular junction. It is likely that these particles represent the sites for known extrajunctional Ach sensitivity in denervated muscle. Work is in progress to test this hypothesis. The observed variation in the size of the aggregates of particles may be related to a gradual and progressive change in the membrane. The significance of the apparent decrease in number of 80 Å particles on face P is uncertain.

Acknowledgements

This work has been supported by grants (to S.K.M.) from the National Research Council of Canada. U.T. is a graduate student in the Department of Zoology.

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Fig.1. (A) Freeze-fractured innervated sarcolemma (control) showing the convex face (P) of the membrane. The intra-membranous 80 A particles are uniformly dispersed over the entire face. Arrow head indicates direction of shadowing in all figures. × 60 000. (B–D) Freeze-fractured sarcolemma from denervated muscle showing various alterations on face P. (B) Shows a marked decrease in number of 80 A particles as compared to those discerned on this face in the control (A). The appearance of aggregates of particles which are 110–180 A is seen in (C) (arrows). Larger aggregates of such particles are seen in (D) (arrows). The face labelled E in (B) is the concave face which shows 300–600 A protuberances representing openings of T-tubules or cortical vesicles. A few 80 A particles are also seen on this face, (B–D). × 60 000.

CHAPTER II

Alterations in the Structure of Sarcolemma in a Denervated Skeletal Muscle

Proc. R. Soc. Lond. B. **203**, 59–68 (1978)
Printed in Great Britain

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Alterations in the structure of sarcolemma in a denervated skeletal muscle

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(*Communicated by J. R. Baker, F.R.S. – Received* 15 *June* 1977 – *Revised* 13 *February* 1978)

[Plates 1–4]

The structure of lumbricals of the rat has been investigated with respect to cholinesterase (ChE) and the known hypersensitivity of sarcolemma to acetylcholine (Ach) upon denervation of the muscle. ChE associated with the junctional sarcolemma undergoes a marked decrease in its activity in denervated muscles as detected by a histochemical reaction at electron microscopical level. This activity is inhibited by BW 284 C51, a specific inhibitor of acetylcholinesterase (AchE). It appears therefore that the histochemical reaction used in this study detects specific ChE, i.e. AchE. The relevance of the decrease in ChE upon denervation to the known molecular forms of ChE is discussed. The structure of the sarcolemma in the region of the neuromuscular junction shows no apparent change in the number of intramembranous particles upon denervation that could be visualized in replicas of the freeze-fractured material examined by transmission electron microscopy, thereby indicating that the intramembranous particles are not directly related to the ChE molecules.

The extra-junctional sarcolemma of the denervated muscle shows 15–18 nm (*ca.* 15 nm) intramembranous particles on the plasmic half of the fractured faces (P face) discernible in electron micrographs of replicas. These particles are often clustered, and the number of particles in these clusters varies from 4 to 50. In contrast, the corresponding fractured face of the contralateral controls shows mostly *ca.* 8 nm particles dispersed throughout the fractured face of the extra-junctional sarcolemma, there being few *ca.* 15 nm particles. On the basis of the known muscle physiology and biochemistry, it is most likely that *ca.* 15 nm particles represent an incorporation of newly synthesized Ach receptors in the extra-junctional sarcolemma after denervation of the muscle. These particles appear to be preferentially attached to the P face of the sarcolemma, there being no depressions on the exoplasmic fractured half (E face) in metallic replicas.

1. Introduction

Vertebrate skeletal muscle provides a valuable model system for investigating the functional organization of cellular membranes. In the differentiated muscle, acetylcholine (Ach) receptors are localized in the subsynaptic sarcolemma. When the muscle is denervated the extra-junctional sarcolemma becomes highly sensitive to Ach (Hartzell & Fambrough 1972); and this hypersensitivity of the muscle results from incorporation of newly synthesized Ach receptors into the extra-junctional sarcolemma (Brockes, Berg & Hall 1975). Biochemical and electron microscopical studies on receptor-rich membranes isolated from the electric organ of fish have indicated that the Ach receptor spans the thickness of the sarcolemma and is exposed at the surface of the membrane (Changeux *et al.* 1975). Such features of the sarcolemma lend themselves to investigation by the freeze–fracturing technique, which enables direct visualization of the interior of cellular membranes (Malhotra & Tewari 1973; Bullivant 1974; Malhotra 1977).

Furthermore, acetylcholinesterase (AchE) in vertebrate muscle is generally known to serve in the termination of nerve impulse transmission by hydrolysis of acetylcholine (Ach) released from the presynaptic nerve ending (reviewed by Silman 1976). Recent biochemical investigations on rat diaphragm muscle indicate that there are three molecular forms of AchE with sedimentation constants of 4, 10 and 16*S*. The last mentioned molecular form is specifically associated with the neuromuscular junction (Hall 1973). It should be mentioned that similar three molecular forms of AchE with somewhat higher sedimentation constant values (8, 14, and 18*S*) have been described from electric organ of electric fish (Silman 1976). AchE has a complex molecular structure in which there is a multisubunit head bearing the active sites of the enzyme attached to an elongated tail. Based upon enzymatic digestion with protease and collagenase, the tail is thought to be collagenous and appears to anchor the enzyme to the basement membrane. AchE from various sources possesses an elongated structure, which appears to be similar in overall features (Silman 1976).

The present study was undertaken with a view to investigate whether the ChE molecules are related with the intramembranous particles in the sarcolemma. Denervation of a skeletal muscle has been employed as an experimental manipulation, since more than 50 % of the ChE activity is lost within 3 days after denervation (Guth, Abers & Brown 1964), and the largest proportional decrease occurs in the 16*S* form of AchE (Hall 1973). The distribution of ChE in innervated and denervated sarcolemma of the skeletal muscle of the rat was studied by using a histochemical reaction suitable for electron microscopy (Karnovsky & Roots 1964). It appears from the distribution of the reaction product that the histochemical test used in this investigation preferentially localizes the ChE in the region of the neuromuscular junction. Furthermore, the results of such histochemical studies considered in conjunction with the freeze–fracture data on innervated and denervated muscle suggest that the intra-

membranous particles seen on the fractured faces of the sarcolemma at the neuromuscular junction are not directly related to the ChE.

A brief account of the structure of the extra-junctional sarcolemma in innervated and denervated muscle is reported elsewhere (Tipnis & Malhotra 1976).

2. Materials and methods

Lumbricals of the hind leg of female rats (100–150 g) of Sprague Dawley strain were used in the present investigation. These particular muscles were selected because they are small and can be easily dissected and fixed *in situ*. In all the experiments on denervation, the left leg was denervated and the contralateral right leg was used for control experiments. The rats were anaesthetized by intraperitoneal injection of 1 ml of sodium pentobarbital at a concentration of 5 mg/ml (approximately 30 mg/kg). The rats were denervated by excision of approximately 1 cm of the sciatic nerve in the thigh region, and killed two weeks after the nerve was sectioned.

(*a*) *Preparation of lumbricals for electron microscopy*

After anaesthetizing the animals 0.5–1.0 ml of 2 % glutaraldehyde, buffered with 0.1 M phosphate at pH 7.2 was injected into the hind paw to fix the lumbricals *in situ* and 10 min later the skin on the ventral side of the paw was excised so that the plantar aponeurosis with its adhering connective tissue could be removed to expose the lumbricals. The latter were removed and further fixed in the glutaraldehyde solution for 2 h. For thin sectioning, some of the lumbricals were further processed by routine fixation in osmium tetroxide, dehydration in ethanol and embedding in Araldite (Tipnis 1977). For freeze-fracture studies glutaraldehyde-fixed lumbricals were soaked in 20 % glycerol and frozen in liquid Freon 22. In order to enhance the chances of obtaining fractures through the neuromuscular junctions, some of the glutaraldehyde-fixed lumbricals were stained for ChE as described below so that the endplate regions could be identified as brownish zones under a dissecting microscope; such regions were cut and glycerolated before freezing in Freon 22. They were fractured at −100 °C in a Balzars 360 M freeze–etch unit and Pt–C replicas made (Tewari & Malhotra 1974; Tipnis & Malhotra 1976). The replicas were cleaned in 40 % chromic acid, washed in distilled water and examined by transmission electron microscopy.

(*b*) *Histochemical staining technique for cholinesterase* (ChE)

After anaesthetizing the rats, ice-cold 0.5 % glutaraldehyde, buffered with 0.1 M cacodylate at pH 7.2, was injected into the hind paw, and the lumbricals removed as described above. They were further fixed in 0.5 % glutaraldehyde solution for a total period of 1 h. After rinsing in cacodylate buffer, the muscles were either chopped at 25–50 μm on a Smith and Farquhar tissue sectioner or were teased into bundles containing 3–7 muscle fibres to facilitate penetration.

Such tissues were incubated in a medium containing the following chemicals: 0.1 % acetylthiocholine iodide, 0.2 M sodium citrate, 0.002 M copper sulphate, 0.0003 M potassium ferricyanide, 0.1 M cacodylate buffer (pH 6.0) (see Karnovsky & Roots 1964; Rash & Ellisman 1974). Eserine at 10^{-6} M concentration was added to the above incubation medium in some of the experiments to inhibit the total ChE reaction (Karnovsky 1964); and 1,5 *bis* (4 allyldimethylammonium phenyl) pentane-3-one dibromide (BW 284 C 51) at 10^{-4} M concentration was used as an inhibitor for specific ChE, i.e. AchE. The incubation in BW 284 C 51 was performed according to the procedure given by Somogyi, Chubb & Smith (1975). The tissue was also incubated in medium from which substrate had been omitted, in order to determine the non-specific binding, if any. The muscle tissues were incubated for 30–45 min, washed in buffer, postfixed in 2 % osmium tetroxide buffered with cacodylate (pH 7.2), and processed for thin sectioning. The thin sections were examined either unstained or stained with uranyl acetate. It should be pointed out that the above histochemical technique for ChE has been widely used at the electron microscope level (Barajas, Silverman & Mueller 1974; Rash & Ellisman 1974; McMahan, Sanes & Marshall 1978).

(c) *Number of experiments carried out*

In each of the control and denervated groupings of rats, the results on thin sections and histochemistry are based upon study of muscles from at least 4 rats, with the exception of experiments on the use of BW 284 C 51 inhibitor which are based on 3 trials. For freeze–fracture study, 72 replicas were prepared from control muscles of 24 rats, and 40 replicas out of 14 denervated rats. Out of these, 50 replicas of control muscle and 20 replicas of denervated muscle were examined.

3. Results

The structure of the lumbrical muscles of the rat is very similar to that of white (fast-twitch) skeletal muscle given by Bloom & Fawcett (1975). The white muscle fibres are distinguishable from the red fibres (slow-twitch) which have abundant mitochondria and ribosomes. White fibres are characterized by the presence of few mitochondria, arranged in pairs or small rows, scanty ribosomes, and are larger in diameter than the red fibres. In the lumbrical muscle of the rat most of the fibres are of white type, there being an occasional red one. The present results are based on the study of a large number of fibres per muscle, and it is therefore assumed that they belong to the white type.

When a skeletal muscle is denervated, it undergoes gradual changes in its structure and by about two weeks the entire sarcolemma becomes sensitive to Ach (Hartzell & Fambrough 1972). Therefore lumbrical muscles from rats which had been denervated for two weeks were used for the present investigation for analysis of the sarcolemma by freeze-fracturing and for histochemical detection of ChE. The degenerative changes in the structure of muscle fibres discernible in

electron micrographs of thin sections are comparable to those already published by various workers (for example Pellegrino & Franzini 1963; Miledi & Slater 1968; see also Tipnis 1977) and therefore such an account is not included in this paper.

(*a*) *Cholinesterase localization*

In electron micrographs of innervated (control) lumbrical muscle subjected to the histochemical reaction, a uniformly dispersed dense deposit is evident throughout the synaptic cleft in the region of the neuromuscular junction (figure 1). (This distribution of the reaction product is invariably found in the region of the neuromuscular junction encountered in all of the thin sections examined.) No reaction product is detectable in association with the sarcolemma in the non-junctional region. Apart from the neuromuscular junction, electron dense deposits are also discerned in association with the plasma membrane of myelinated axons and occasionally in the sarcoplasm. This variable occurrence of the reaction product in the sarcoplasm is evident irrespective of whether the histochemical reaction is carried out on teased muscle fibres or on 25–50 μm sections. Such a variation may result from lack of penetration through the muscle by reagents in the incubation medium. In view of this apparent difficulty in consistently demonstrating the ChE activity in the sarcoplasm, results of our studies on the use of inhibitors for ChE cannot be interpreted in a meaningful way in respect to the nature of ChE in the sarcoplasm. No further remarks are consequently feasible on the distribution of ChE in locations other than at the neuromuscular junction and sarcolemma.

In lumbricals of the rat denervated for 2 weeks and then subjected to the histochemical reaction for ChE, the electron dense deposits are only found in the synaptic cleft. But in contrast to the rather uniform distribution in this location in the innervated muscle, the denervated muscle shows a non-uniform distribution of electron dense deposits. This distribution varies in different neuromuscular junctions examined (figures 2 and 3) and neuromuscular junctions totally devoid of electron dense deposits are also seen in denervated rats (figure 4). It is emphasized that neuromuscular junctions totally devoid of any electron dense deposits are seen in denervated rats (figure 4). This variation in localization of ChE activity does not seem to be due to discrepancy in technical variation since all the neuromuscular junctions visualized in innervated muscle showed a uniform distribution of electron dense deposits throughout the synaptic cleft. It is likely therefore that the variation in the reaction product in denervated muscle reflects an underlying varying decrease in the ChE activity along the synaptic cleft in different muscle fibres.

When the histochemical reaction is carried out in the presence of eserine, or BW 284 C 51 no reaction product is discerned in the synaptic cleft of denervated or innervated rats (figures 5 and 6). Also, when the histochemical reaction is carried out in the absence of the substrate no electron dense deposit is discerned in association with any structure in muscle thereby eliminating the possibility of non-specific binding of constituents in the incubation medium.

In view of the above findings, it is tentatively concluded that the electron dense deposits in the synaptic cleft of innervated muscle represent localization of specific ChE, i.e. AchE, and that this activity undergoes a marked decrease upon denervation of the muscle.

(*b*) *Freeze-fracture studies*

The fractured faces of the sarcolemma are labelled as face P or face E corresponding to whether the fractured membrane is adjacent to the sarcoplasm or the extracellular space respectively. This nomenclature is in accordance with the criteria proposed by Branton *et al.* (1975). The complementary fractured halves of most biological membranes appear asymmetrical in respect of the distribution of number of particles, there being more particles on P face (see Malhotra & Tewari 1973; Bullivant 1974; Stoeckenius 1976; Malhotra 1977).

(i) *Sarcolemma in the region of the neuromuscular junction*

It should be emphasized that large areas of the fractured sarcolemma in the neuromuscular zone have not been easy to detect in the replicas. This is perhaps due to the inability of the fracture plane to follow through the deeply invaginated sarcolemma in this region. However, the identification of the fractured faces has been facilitated by the presence of presynaptic endings in cross sections. In the innervated muscle the P face of the postsynaptic membrane is characterized by the presence of 15–18 nm particles which are concentrated in the upper region of the junctional folds. Their packing density is approximately 1800/μm^2, which is comparable to that described for this face in extensor digitorum longus, gastrocnemius and diaphragm muscles of the rat (Rash & Ellisman 1974). Particles 8 nm in size are found only in the deeper regions of the junctional folds. This face can also be distinguished from the comparable face of the extra-junctional sarcolemma by the absence of depressions corresponding to the T-system or caveolae seen in the the extra-junctional sarcolemma. The E face of the postsynaptic sarcolemma has a pitted appearance and very few particles (figure 7). Also, protuberances (30–50 nm) which are present on the corresponding fractured face of the extra-junctional sarcolemma are absent. There are no depressions on the E face that

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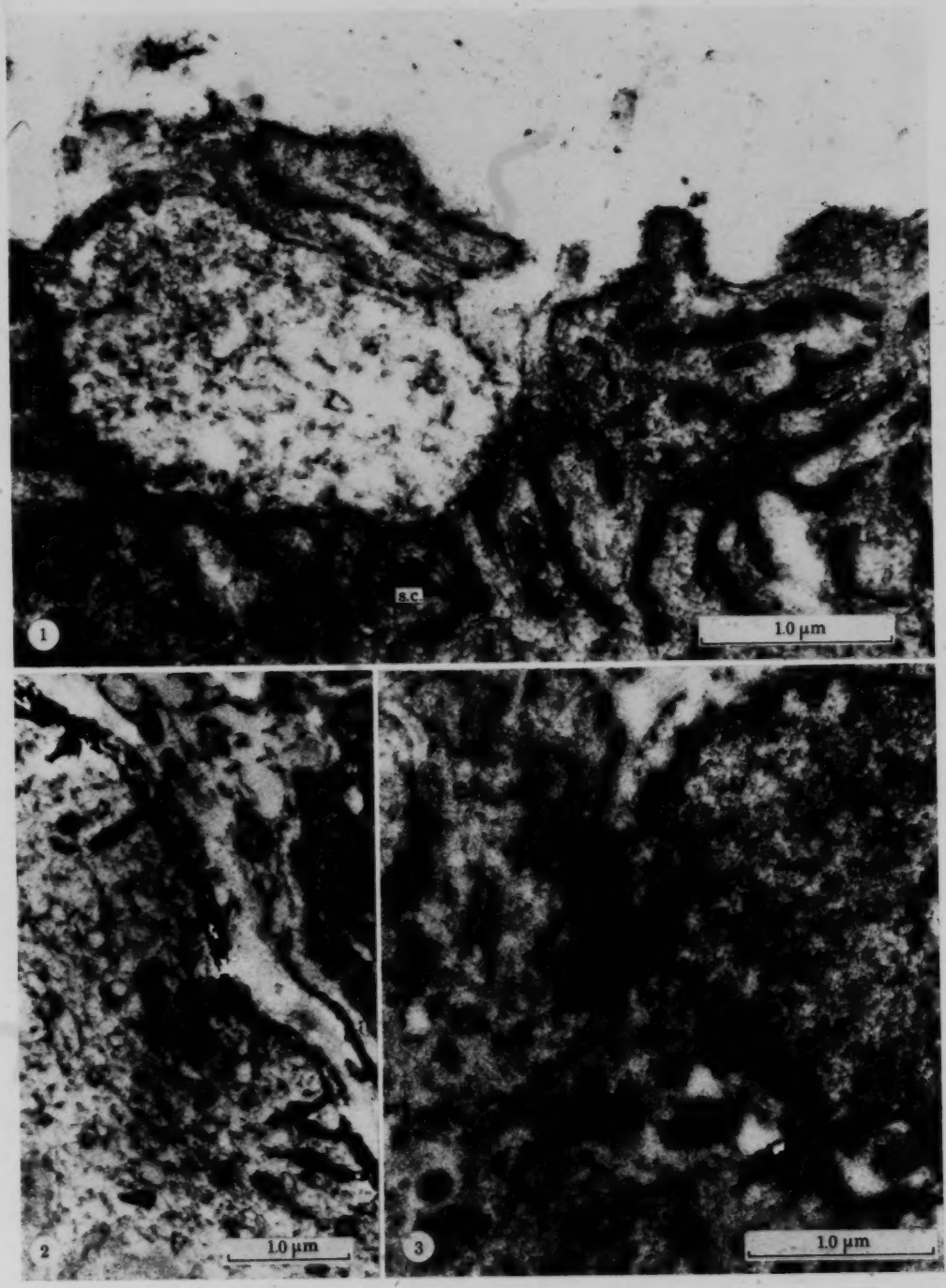
Description of plates 1 and 2

Figure 1. Lumbrical muscle of innervated (control) rat subjected to histochemical reaction for cholinesterase (ChE) showing electron dense reaction product in the synaptic cleft (sc.).

Figures 2–4. Lumbrical muscle of the denervated rat subjected to histochemical reaction for cholinesterase, showing either a patchy appearance of the electron dense reaction product (arrow heads in figures 2 and 3), or no positive reaction at all, in the neuromuscular junctions encountered in the denervated muscle (figure 4).

Figures 5 and 6. Lumbrical muscle from innervated rat subjected to this histochemical reaction for cholinesterase in the presence of eserine (figure 5) or BW 284 C51 (figure 6) to inhibit the enzymatic reaction. No electron dense reaction product is discernible in association with any of the cellular structures encountered in thin sections of the muscle.



FIGURES 1–3. For description see opposite.

(*Facing p.* 64)

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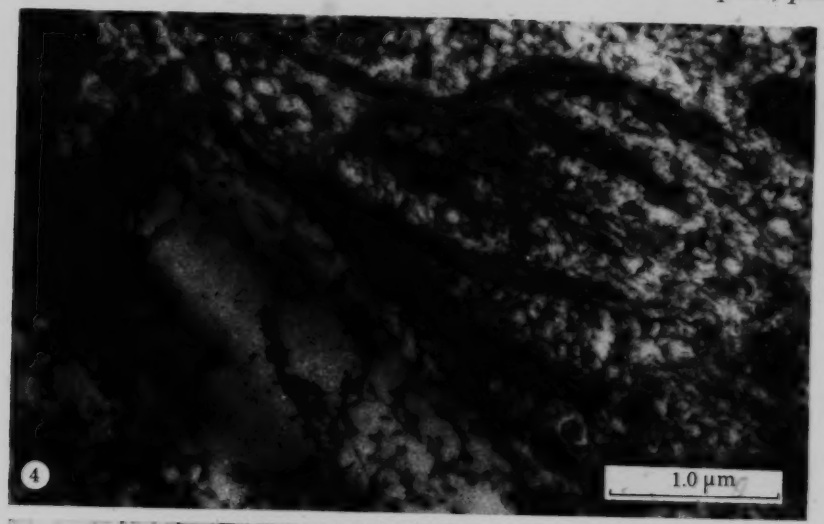
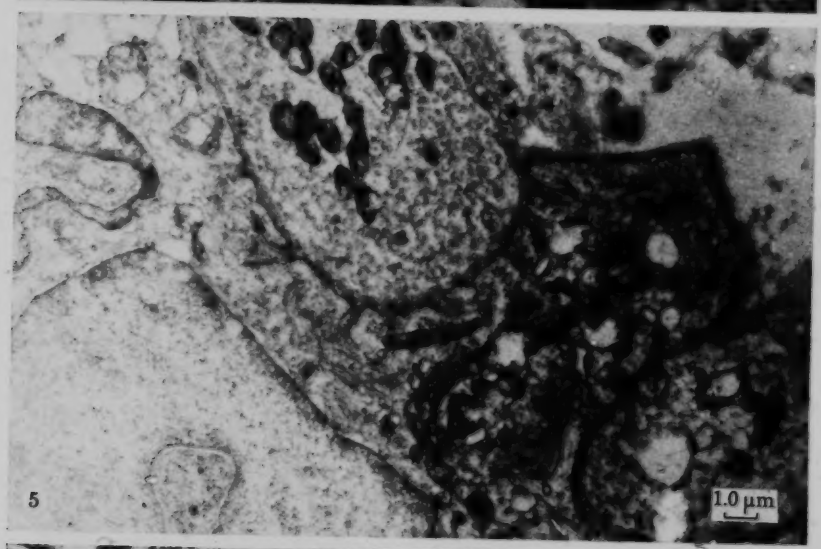
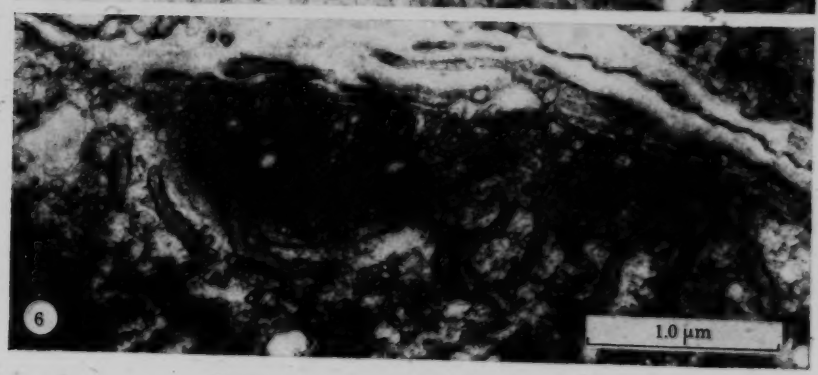
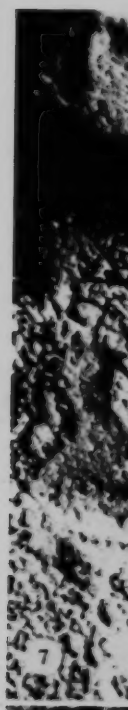
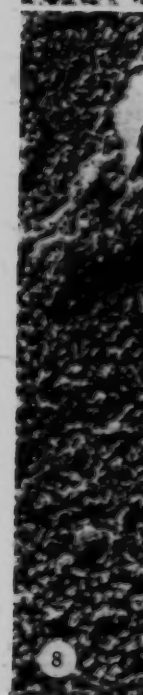


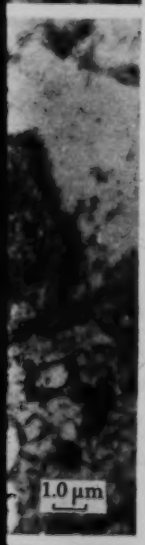


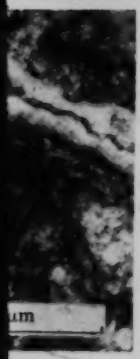


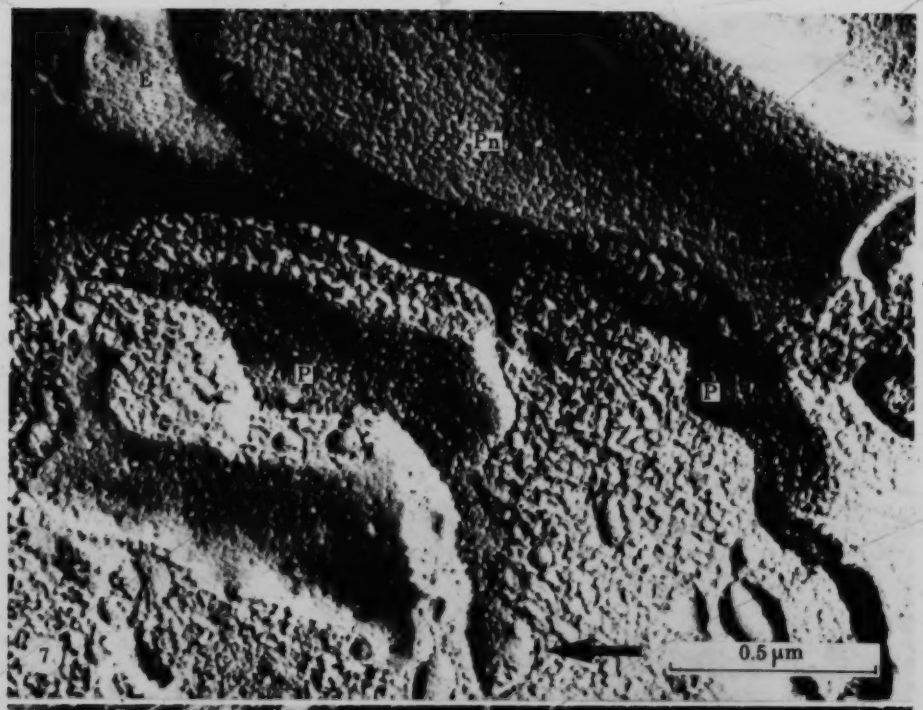
FIGURES 4–6. For description see p. 64.



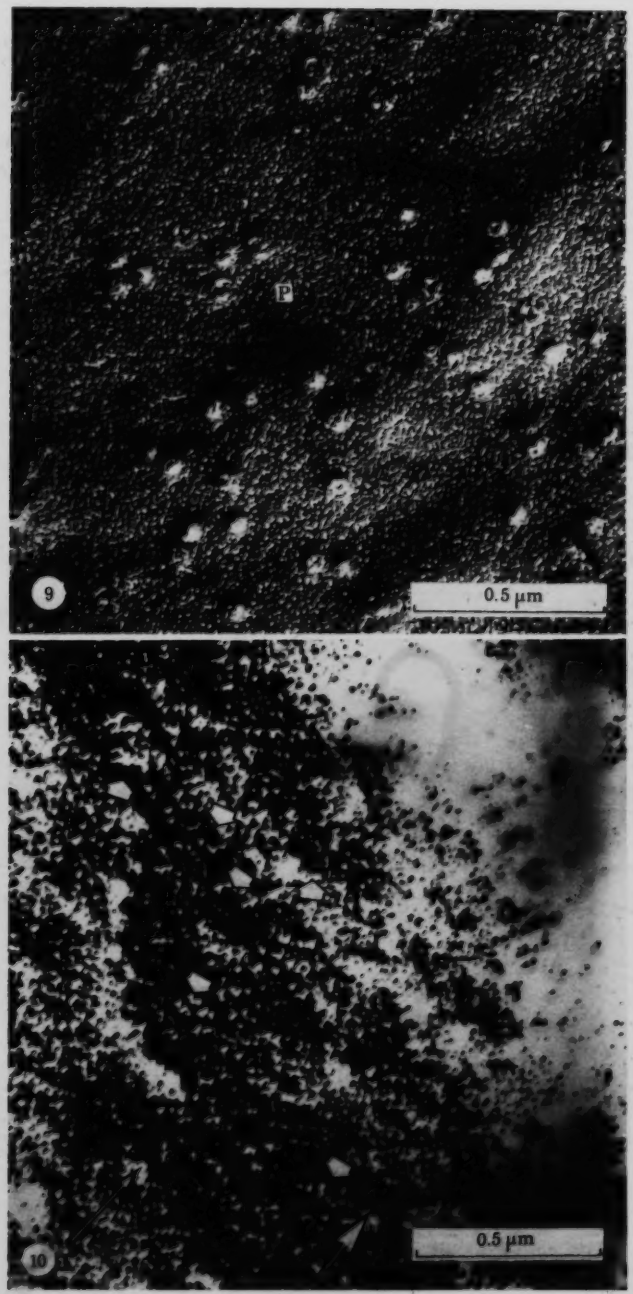








FIGURES 7 AND 8. For description see p. 65.



FIGURES 9 AND 10. For description see opposite.

correspond to the particles on the complementary face. This asymmetry in the structure of the sarcolemma may arise from a preferential attachment of the particles to the P face (Stoeckenius 1976).

There are no discernible alterations with respect to the number of intramembranous particles on the P or E face of the sarcolemma in the neuromuscular junctional zone when the muscle is denervated for 2 weeks (figure 8).

(ii) *Sarcolemma in non-junctional zone*

The two complementary fractured faces of the extra-junctional sarcolemma appear asymmetrical in respect of the distribution of intramembranous particles there being far fewer particles on E face than are found on the P face (figure 9). These particles are mostly 8 nm in size and rather uniformly dispersed. There are approximately 2000 particles/μm^2 on the P face. The P face also shows a large number of depressions 30–50 nm in size, which most likely correspond to the distribution of T-tubules or cortical vesicles. The E face shows protuberances which correspond in size and distribution to the 30–50 nm depressions on the complementary P face.

The most conspicuous features of the P face of the extra-junctional sarcolemma of the denervated muscle is the presence of intramembranous particles 15–18 nm (*ca.* 15 nm) in size. These particles are found either dispersed at random throughout the fractured face or more often occur in aggregates. There are varying numbers of particles in each aggregate, from 4 to 50 (figure 10). It is emphasized that there is a large variation in size and number of aggregates that are found on the P face in the replicas. However, the 15–18 nm particles and their occurrence in aggregates is characteristic of the extra-junctional sarcolemma of the denervated muscle because these features are not apparent on the corresponding fractured face of the innervated muscle. Further, the 8 nm particles that are

Description of plates 3 and 4

Figures 7 and 8. Fractured faces of the sarcolemma in the region of neuromuscular junction of control (figure 7) and denervated (figure 8) muscle showing essentially similar number of intramembranous particles on P face of the subsynaptic sarcolemma in both types of muscle. Also, E face has fewer particles than on its complementary P face. Pn, P face of nerve terminal. Arrow, lower right, shows direction of shadowing.

Figure 9. Typical structure of the convex fractured (P) face of the sarcolemma in the extra-junctional region of the innervated lumbrical muscle. This face is characterized by the presence of a large number of 8 nm particles and large depressions scattered throughout. Arrow, lower right, shows direction of shadowing.

Figure 10. P face of the sarcolemma of the lumbrical muscle from a denervated rat, showing aggregates of *ca.* 15 nm particles (arrow-heads) that are not obvious on the comparable face of the control (figure 9). Also note that there are fewer 8 nm particles than in figure 9. Arrow, lower right, shows direction of shadowing.

abundant on the P face of the extra-junctional sarcolemma of the control muscle are far fewer in the denervated muscle.

The E face of the extra-junctional sarcolemma of the denervated muscle appears similar to that of the comparable face of the control muscle. No groups of depressions complementary to the aggregates on the P face have been found on the E face. This asymmetry in the structure of the complementary fractured faces could well result from a preferential attachment of the particles to the P face as has been described for the particles representing bacteriorhodopsin in the purple membranes of halobacteria (Stoeckenius 1976) also referred to above (p. 64)

4. Discussion

The histochemical reaction used in the present investigation appears to localize preferentially specific ChE (AchE) in the cleft of the neuromuscular junction, because a specific inhibitor for AchE (BW 284 C 51) abolishes the histochemical reaction in this region. It is relevant that Rash & Ellisman (1974) and McMahan *et al.* (1978) have reported that ChE activity is associated with the basal lamina in the region of the neuromuscular junction in vertebrate skeletal muscle. Their conclusions are based upon the application of Karnovsky's histochemical technique employed for the present study. It is speculated that the histochemical reaction used for the present investigation localizes the 16*S* form of AchE. This assumption is based upon the known distribution of the three molecular forms of AchE (see Introduction); 4*S* and 10*S* forms of AchE have been reported to be distributed throughout the muscle (Hall 1973) and histochemical reaction product corresponding to such a distribution is absent from extra-junctional sarcolemma of innervated muscle. Quantitative studies on denervated sternomastoid muscle of the rat have shown that ChE activity decreases between 50 and 70 % within 3 days after denervation, and very little change occurs thereafter (Guth *et al.* 1964). The decrease observed in the histochemical reaction product in the synaptic clefts in denervated muscle is consistent with the findings of Hall (1973) that the largest proportional decrease occurs in 16*S* form of AchE in denervated muscle. The kinetic behaviour of each of the three species of AchE showed that both 16*S* and 10*S* AchE exhibit substrate inhibition at Ach concentrations above *ca.* 1.25 mM. The 4*S* form, however, showed no inhibition of activity with Ach concentrations up to 10 mM, which distinguishes the 4*S* form of AchE from 16*S* and 10*S* forms. The active hydrolysis of β-methylacetylcholine by the 4*S* form and its inhibition by the specific inhibitor BW 284 C 51 clearly demonstrate it to be an acetylcholinesterase (Hall 1973). While it is conceivable that a particular molecular species of AchE, i.e. 16*S*- is functional at the neuromuscular junction, the precise rôle served by the other forms of AchE remains to be investigated. A suggestion has been made that they may be intermediates in biosynthesis and assembly, different functional forms at separate loci, or aggregates and degradation products produced as artefacts of biochemical processing (Silman 1976).

A comparison of the structure of the freeze-fractured faces of the post-synaptic membrane of the neuromuscular junction in innervated and denervated rats leads to the obvious conclusion that the intramembranous particles are not directly related to the AchE molecules. This conclusion is based on the observation that the number of intramembranous particles on the P face of the synaptic sarcolemma does not apparently change in the muscle denervated for 15 days, while the AchE shows a marked decrease in the denervated muscle. It is recalled that the non-junctional sarcolemma undergoes marked alterations in the structure of the fractured faces in denervated muscle. This change is reflected in the increase and distribution of *ca.* 15 nm particles, which, it is hypothesized, are the Ach receptor molecules (Tipnis & Malhotra 1976). It is likely that the aggregates of 15 nm particles observed on the P face of the extra-junctional sarcolemma correspond in distribution to the discrete patches of high Ach receptor density reported in the denervated skeletal muscle of mice by using fluorescent conjugated α-bungarotoxin and ^{125}I-α-bungarotoxin (Ko, Anderson & Cohen 1977). Also the size of the particles in the aggregates found on the P face of the denervated muscle is consistent with that reported for Ach receptors in neuromuscular junctions from a variety of mammalian muscles (Rash & Ellisman 1974). It is likely that these particles represent the sites for known extra-junctional Ach sensitivity in the denervated muscle. Work is in progress to test this hypothesis. The observed variation in the size of the aggregates of *ca.* 15 nm particles may be related to a gradual and progressive change in the membrane. The significance of the apparent decrease in the number of 8 nm particles on the P face is not known.

This work has been supported by grants (A5021) from the National Research Council of Canada, and a General Research Grant from the University of Alberta. The technical assistance of Harold Batz is greatly appreciated.

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CHAPTER III

α-Bungarotoxin Binding Sites (Acetylcholine Receptors) in Denervated Skeletal Muscle of the Rat

(U. R. Tipnis and S. K. Malhotra)

J. Supramol. Structure, in press

ABSTRACT

The non-synaptic sarcolemma of denervated skeletal muscle of rat shows an abundance of ~15nm intramembranous particles on the P-face. These particles are either singly distributed or are in clusters, and they are missing from the comparable freeze-fractures of the innervated sarcolemma. Autoradiographic studies by using ^{125}I-α-BGT on 1μm thick sections and freeze-etch studies by using ferritin-α-BGT conjugates on membrane fractions, show that the distribution of the label corresponds to the distribution of the 15nm particles in the non-synaptic sarcolemma. On the basis of these results and existing physiological and biochemical data, it is suggested that the 15nm intramembranous particles are components of the α-BCT binding sites, i.e., acetylcholine (Ach) receptors, in the non-synaptic sarcolemma of denervated muscle and that the two types of distribution represent two spatial manifestations of Ach receptor molecules. The significance of these findings in relation to synapse formation in denervated muscle is discussed.

Key Words: Denervated sarcolemma; non-synaptic acetylcholine receptors; ^{125}I-α-bungarotoxin; ferritin-α-bungarotoxin; electron microscopy; freeze-fracture etching; autoradiography.

INTRODUCTION

Denervation of the adult mammalian skeletal muscle is being extensively applied to understanding the nerve-muscle interaction (*1,2,3,4, 5,6*). Physiological and biochemical changes occurring in the sarcolemma of denervated muscle include a fall in the resting membrane potential and an increase in membrane resistance (*7,8*) and a decrease in acetylcholinesterase activity (AchE), mainly the 16s form of AchE (*6,9*). Amongst the three known molecular forms of AchE, namely 4s, 10s and 16s in mammalian muscle, the induction of the 16s form is under neural control (*10*). Denervation also results in an increased sensitivity to acetylcholine (Ach) in non-synaptic sarcolemma with a concomitant increase in the number of Ach receptors (*11*). Earlier, we reported changes in the sarcolemma of denervated rat muscle examined by freeze-fracturing technique (*4*) and described the appearance of intramembranous particles (~15nm) on the cytoplasmic fracture of the membrane (P-face) in the non-synaptic sarcolemma. These intramembranous particles were either singly distributed or in clusters. It was suggested that these ~15nm particles which appear in the non-synaptic region following denervation are the Ach receptors. The present paper describes the work primarily directed towards testing the above suggestion by use of marker, α-bungarotoxin (α-BGT), which binds to the Ach receptors in a specific

and irreversible manner (*12*). α-bungarotoxin, when conjugated to ferritin, can be visualized by electron microscopy or, when conjugated to ^{125}Iodine, can be demonstrated by autoradiography at light or electron microscope level. The results of such an investigation are included in this paper.

It should be pointed out that in mammalian muscle the size of intramembranous particles thought to correspond to the Ach receptor complex is 11-15ηm (*6,37*). In *Xenopus* embryonic muscle and cultured myotubes of chick, such particles are 10-19ηm (*38,39*). These sizes differ from the 7ηm given for the Ach receptor complex in the electroplaques (*40*). Though the significance of these differences remains to be investigated, the intramembranous particles in the segments of cultured myotubes of chick containing identifiable regions of high acetylcholine sensitivity measure 10-19ηm (*39*).

MATERIAL AND METHODS

Denervation Procedure

The lumbricals and extensor digitorum longus (EDL) of rats of the Sprague Dawley strain and weighing 100-120 gms were used in this study. The animals were anaesthetised and denervated by transection of the sciatic nerve in the upper thigh region and sacrificed by cervical

decapitation 2 weeks after denervation. The rationale for using such a period of denervation was based upon our previous morphological and histochemical studies which showed marked changes in the non-synaptic sarcolemma after 2 weeks of denervation (*4*,*6*). Also, the incorporation of Ach receptors in the non-synaptic sarcolemma is optimum 2 weeks after denervation (*11*). The muscles of normal innervated rats were used as controls.

Preparation of Muscle Membrane Fraction (see Appendix I)

The muscles were homogenized and crude membrane fractions were isolated from control, as well as denervated animals, according to the procedure of Boegman *et al.* (*13*).

Preparation of α-bungarotoxin (α-BGT) Conjugates

α-BGT supplied by Miami Serpentarium, Florida, was used for the following conjugates:

(a) Iodination of α-BGT

α-BGT was iodinated with 1 mCi ^{125}I using the method of Greenwood and Hunter (*14*). The iodination was carried out by Radiopharmacy Centre, University of Alberta. The specific activity and protein concentration of iodinated protein were 2.192×10^5 Ci/M and 8.4 μg/ml respectively.

(b) Preparation of ferritin-α-bungarotoxin (Ft-α-BGT) (Appendix II)

Ferritin (6x crystallised and cadmium free) obtained from Polysciences was conjugated to α-BGT by using glutaraldehyde, according to the method of Hourani *et al.* (*15*; see Appendix II for details of preparation).

Incubation of Muscles With ^{125}I-α-BGT

The EDL muscles from denervated and innervated rats were tied at both ends to a wooden stick and immediately transferred to an oxygenated Kreb's ringer containing ^{125}I-α-BGT (2×10^{-7} M). The muscles were incubated for 2 hours. For determining the specific binding of α-BGT, another set of EDL from each of the innervated and denervated rats was incubated initially in Kreb's ringer containing d-tubocurarine (10^{-4} M) for 1 hour, followed by incubation in ^{125}I-α-BGT for 2 hours. During incubation, the oxygen was continuously bubbled through the medium maintained at 37°C in a water bath. After incubation, the muscles were thoroughly washed by repeated changes of buffer during 1 hour and fixed in 2% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2). The tissue was then given several changes of buffer and gently dried on filter paper and weighed. The radioactive counting of these tissues was done in a gamma counter (Baird-Atomic). The tissue was then post-fixed in OsO_4 and routinely processed for preparation of 1μm thick Araldite sections for autoradiography (see below).

Incubation of Membranes in ^{125}I-α-BGT (see Appendix III)

The membranes were assayed for their Ach receptor activity by using the filter assay procedure suggested by Klett *et al.* (*16*; see page 154, Appendix III): 1 ml

of Kreb's ringer contained 50 µg of membrane protein, ^{125}I-α-BGT (2 x 10^{-9} M) 1% (W/V) Tween 80. Control samples were incubated with α-BGT (10^{-4} M) for 1 hour prior to incubation in ^{125}I-α-BGT. The mixture was incubated at room temperature (20-22°C) for a varying period and then filtered through DE81 cellulose anion exchange filter disc (Whatman). The dried filter discs were counted for the radioactivity in a gamma counter.

Preparative Procedures for Microscopy

(a) Light microscope autoradiography

1µm thick Araldite sections were cut from blocks of innervated and denervated muscles which had previously been incubated in ^{125}I-α-BGT as described above. The sections were coated with 1:1 diluted Ilford L_4 emulsion on gelatinized slides. After exposure for one week, the slides were developed and fixed in 25% sodium thiosulphate. The sections were stained with 2% phenylenediamine and examined under a phase contrast microscope.

(b) Incubation of muscle membranes with Ft-α-BGT

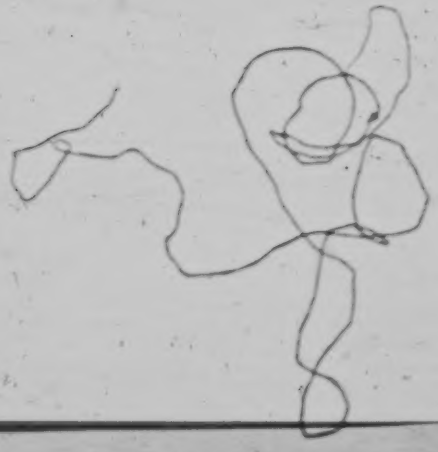
The muscle homogenate (1000 g) and the crude membrane fractions were incubated with Ft-α-BGT (0.6 µg/ml) for 1 hour. For determination of specific binding, the fractions were first incubated in α-BGT (10^{-4} M) or d-tubocurarine (10^{-4} M),

followed by incubation in the conjugate. The suspension of crude membranes was washed by filtration through millipore as described by Karlin *et al.* (*17*).

Suspension of the homogenate was centrifuged at 1000 g for 10 minutes. The supernatant was discarded and the pellet was washed several times with buffer. The samples were then fixed and processed for electron microscopy as described above. Thin sections were examined, unstained or stained with uranyl acetate and lead citrate, in Phillips EM 300 electron microscope.

(c) Freeze-fracture and freeze-etch preparations

A portion of the homogenate incubated in Ft-α-BGT was used for freeze-etching. The pelleted samples were fixed in 2% glutaraldehyde buffered with 0.1 M phosphate (pH 7.2). The material was rinsed with buffer and finally with distilled water. Freeze-fracturing and etching were done in Balzers BA 360M and the etching period was up to 2 minutes. The replicas were washed in 40% chromic acid and rinsed in several changes of water and examined in Phillips EM 300 electron microscope.



RESULTS

Freeze-fracture studies of innervated and denervated sarcolemma

A comprehensive account of the alterations in the structure of sarcolemma visualized in the freeze-fractured replicas of denervated muscle is given elsewhere (*4,5,6*); therefore, only those features which are pertinent to the present investigation are mentioned below.

In innervated non-synaptic sarcolemma, the PF shows randomly dispersed intramembranous particles which are approximately 8 nm in diameter ($\sim$2000/μm^2) (Fig. 1A). In contrast, the corresponding face of the denervated sarcolemma shows an abundance of $\sim$15 nm (15-18 nm) particles. These particles are dispersed singly or in aggregates. These aggregates may be small with as few as 4-10 particles or large with approximately 25-100 particles (Fig. 1B). The average density of the particles on the PF (convex fracture) is $\sim$400 particles/μm^2 and is predominantly made up by the 15nm particles. A precise correlation between the intramembranous particles and the Ach receptors remains to be determined. It is of interest, however, that there are $\sim$1150 α-BGT binding sites/μm^2 in the denervated extrajunctional sarcolemma. This estimate is based upon the assumption that each receptor binds one ^{125}I-α-BGT molecule (Tipnis and Malhotra, unpublished data), yet there may be more than one binding site per receptor molecule (*43*). On the basis of existing physiological and

biochemical evidence (*11,18*), it has been hypothesized that these particles are components of the α-BGT binding sites (Ach receptors, *4,5*) and the two types of distributions of particles noted in these freeze-fractured replicas represent two distinct topographic distributions of receptors in the non-synaptic sarcolemma of denervated muscle.

The following results refer to the experiments designed to test the above hypothesis by localization of Ach receptors through the use of ^{125}I-α-BGT and Ft-α-BGT conjugates.

Incorporation of ^{125}I-α-BGT into muscle

Incubation of innervated and denervated muscles in media containing ^{125}I-α-BGT shows a marked increase in the binding of toxin by denervated muscle over the innervated muscle (Table 1). Pre-incubation of the muscles with d-tubocurarine leads to a marked suppression in the binding of the toxin (Table 1). Since d-tubocurarine is known to be a specific ligand for Ach receptors (*19*) and binds to the same site as the α-BGT, the data on the specific incorporation of ^{125}I-α-BGT indicate the labelling of the Ach receptors. As seen in Table 1, the specific incorporation of ^{125}I-α-BGT in denervated muscle is approximately 4 fold more than that in the innervated muscle. In both innervated and denervated muscle, d-tubocurarine inhibits toxin labelling by about 50%, which is in agreement with previous findings (*41,42*).

Light microscope autoradiography

Table 2 shows the number of silver grains seen in the non-synaptic sarcolemma of 1µm thick sections. Silver grain counts given are after subtraction of the background grains. In each experiment, the background grains have been counted in areas located approximately 5µm away from the tissue. The background counts in sections of ^{125}I-α-BGT labelled muscle are comparable to the background grains encountered in sections prepared from muscles incubated in cold α-BGT and processed for autoradiography.

The distribution of silver grains in 1µm thick sections from innervated and denervated muscle is displayed in Figure 2. The silver grains in the non-synaptic sarcolemma of denervated muscle appear either dispersed singly (Fig. 2C) or clustered (Fig. 2B). The number of silver grains per micrometer square is approximately 5-10 fold higher in the clusters than outside such regions. Such areas of higher density do not represent the synaptic regions where the density of silver grains is far more in innervated as well as denervated muscle (Fig. 2A).

In contrast, the non-synaptic regions of the innervated muscle have very few grains and their number is close to the background density (Fig. 2D). Also, sections of denervated muscle treated with d-tubocurarine prior to incubation in ^{125}I-α-BGT are generally lacking in silver grains (Fig. 2E). It is concluded from the above data that the silver grains in the non-synaptic sarcolemma of denervated muscles are far more numerous and represent much more binding of ^{125}I-α-BGT to specific sites on denervated muscle as compared to the corresponding regions of the innervated muscle.

Incorporation of ^{125}I-α-BGT into crude membrane fractions

The presence of sarcolemma in the crude membrane fraction was ascertained by assaying for Ach receptor activity. [Though the Ach receptors have been reported to reside in the Golgi apparatus during

synthesis (*20*), it is assumed that in intact cells the receptors are exposed at the surface only in the plasma membrane.] In the membrane fractions from both innervated and denervated muscle, the binding of the toxin is linear during the first ten minutes, after which saturation occurs (Fig. 3A). The specific activity in denervated membranes is $2.99/10^{-2}$ pmol/μg as compared to 9.2×10^{-4} pmol/μg in innervated preparation. Figure 3B shows that the binding of ^{125}I-α-BGT to denervated membranes is specific, as it is greatly inhibited by preincubation with cold α-BGT.

Transmission electron microscopy of membranes incubated with Ft-α-BGT

Crude membrane fractions show membrane vesicles ranging from 0.2-2μm in diameter. Many of these vesicles from preparations incubated in Ft-α-BGT conjugate and filtered through millipore get trapped in the filter along with non-specifically bound ferritin as reported by Karlin *et al.* (*17*). In the present study, however, vesicles trapped in the filter were not considered and only vesicles lying above the filter were examined. Denervated crude membranes incubated in ferritin conjugate show ferritin associated with the membrane of the vesicle. Ferritin particles may be situated slightly removed from the surface of the membrane (∿5-7nm). The particles are seen as single molecules bound to the membrane or in small clusters (Fig. 4A). Several vesicles

without the associated ferritin molecules are also encountered in electron micrographs. Estimation of the vesicles from four experiments indicates that 60% of the vesicles are labelled (Table 3A) whereas, in d-tubocurarine treated controls, the number of vesicles showing associated ferritin is reduced to 11% of the total vesicles (Table 3B). Small dense particles are sometimes encountered inside the vesicles, both in the experimental as well as the d-tubocurarine treated material. These are generally smaller than the ferritin molecules and their nature is not known. It appears that these particles do not result from preparative fixation procedure, as membrane vesicles which have not been incubated in medium containing Ft-α-BGT conjugate do not show these particles. It is therefore assumed for the present that they represent degraded ferritin molecules, and the membranes are leaky to these particles.

Freeze-etching of Ft-α-BGT labelled and unlabelled homogenate of denervated muscle

Initially, experiments were conducted on Ft-α-BGT labelled crude membrane preparation, but despite several (40-50) attempts, satisfactory fractures of such membranes were not obtained. Therefore, muscle homogenate was investigated as a source of Ach receptor containing membranes. The results reported in this section are based on studies on Ft- -BGT labelled homogenate.

Replicas of freeze-etched membranes in muscle homogenate incubated

with Ft-α-BGT show vesicles with a fractured face (PF) and an etched face (ES). The etched face often displays densely packed bumps (Fig. 5A) comparable to the size of ferritin molecules discernible in metallic replicas (*21*). Some of these bumps are larger than individual ferritin molecules and may represent clusters of ferritin. It is emphasized that only some of the vesicles show bumpy etched face, whereas others have relatively smooth etched face. Also, comparable membrane faces in replicas of the homogenate without incubation in the Ft-α-BGT show relatively smooth etched face (Figs. 5B, 5C). It is therefore concluded that the bumps on the etched face of the membranes displayed in Figure 5A represent ferritin particles presumably bound to the Ach receptors. A comparison of the number of labelled and etched vesicles with the number of labelled vesicles in thin sections would be valuable but the etched vesicles do not occur as often as the labelled vesicles in thin sections. This discrepancy is presumably due to difficulties in getting large areas of etched vesicles in replicas.

It should be remarked that membrane vesicles with scanty intramembranous particles on the fractured face (PF) are seen in the replicas. It is conceivable that such fractured faces represent regions of the membrane that are deficient in particles. Alternatively, they may represent inside-out vesicles and the two fractured faces show an asymmetric distribution of intramembranous particles, there being a few on one half (*22*).

DISCUSSION

The results from autoradiography of ^{125}I-α-BGT and labelling with Ft-α-BGT lead to the conclusion that the Ach receptors are either distributed singly or clustered in the non-synaptic sarcolemma of denervated muscle.

The experiments on ^{125}I-α-BGT binding were primarily undertaken to ascertain the specificity of incorporated radioactivity in both muscle and crude membrane fractions (Figs. 3A, 3B; Tables 1, 2). The filter assay of Klett *et al.* (*16*), employed in the present study, is based on the use of anion exchange cellulose filter for filtering the Ach receptor preparation. The filter binds anionic molecules while ensuring the elimination of cationic substances like unbound α-BGT. In both muscle, as well as the crude membrane fraction from denervated animals, there is a marked increase in incorporated radioactivity. This binding, which is inhibited both by d-tubocurarine and cold α-BGT, is considered specific. The qualitative data based on light microscope autoradiography demonstrates the increased labelling in extrajunctional regions and supports the quantitative data reported in Table 1. These findings, therefore, are in agreement with several physiological studies which have demonstrated the extrajunctional sensitivity to acetylcholine in denervated muscle (*18*,*11*,*23*). This extrajunctional sensitivity results

from the incorporation of newly synthesized receptors (*24,25,26*).

The findings on localisation of Ach receptors, by using ^{125}I-α-BGT and Ft-α-BGT at the level of light and electron microscopy respectively, indicate that there are two distinct populations of Ach receptors, viz., singly dispersed and in clusters. The labelling of receptors with Ft-α-BGT is never as dense as that observed in Ach receptor rich preparation from *Torpedo* electroplaque (*17*). This difference, however, is likely as there is an extremely high concentration of Ach receptors in *Torpedo* electroplaque (40,000-50,000/μm^2) (*27*) as compared to the relatively low concentration (1,695 receptors/μm^2) in the non-synaptic regi n of denervated sarcolemma (*11*). The membrane vesicles from synaptic sarcolemma, which would be expected to show dense labelling of Ft-α-BGT, may not have been encountered and possible explanations for the difficulty in finding such vesicles are: (1) membrane vesicles representing the synaptic sarcolemma are few and are lost during isolation and (2) the membrane preparation is filtered through millipore which traps unbound ferritin as well as vesicles.

The ferritin molecules may appear to be slightly removed from the surface of the membrane in thin sections (Fig. 4A) and this is consistent with the recent studies on Ach receptor rich membranes from *Torpedo* in which it has been reported that the receptor molecules traverse the membrane and project approximately 5.5nm outside the membrane bilayer (*28*).

The presence of Ach receptors in the non-synaptic sarcolemma is a property of adult denervated muscle, as well as that of developing muscle cell (*29*,*30*). The non-uniformity of Ach receptor distribution reported in the present study has also been demonstrated in uninnervated developing muscles from chick and *Xenopus laevis* (*31*,*32*,*33*). The presence of Ach receptor clusters in denervated muscle suggests that in relation to sarcolemma and Ach receptors, the muscle cell reverts to its embryonic state. Ach receptor clusters were thought to be the site of synapse formation (*31*). It is pertinent to mention in this regard that the formation of synapse beside the original end-plates is known to occur either by muscle injury or by removal of a portion of the muscle with the original nerve (*34*,*35*). However, recently Anderson and Cohen (1977) have followed the distribution of Ach receptors (labelled with α-BGT) during synapse formation on myocytes cultured from *Xenopus laevis*. Their studies indicate that the clusters of Ach receptors are not the site of synapse formation.

The significance of the clustered Ach receptors is not yet clear. Axelrod *et al.* (*36*) found that the receptors in the clusters are not free to diffuse into the surrounding areas. It is not as yet clear whether cluster formation occurs by movement and aggregation of individual particles or if there are distinct sites on the membrane where bulk incorporation of Ach receptor molecules takes place. Studies are

in progress to further elucidate the role of the clustered Ach receptors in mammalian skeletal muscle.

ACKNOWLEDGEMENTS

Mr. C. Saunders has provided invaluable technical assistance in the preparation of the material for electron microscopy. We are grateful to Dr. G.W. Stemke for his help in the initial stages in iodination and for the use of his gamma counter. This investigation has been supported through grants awarded by the Natural Science and Engineering Research Council of Canada (A 5021).

Fig. 1A. Shows a freeze-fractured preparation of a normal lumbrical muscle, showing the convex fractured face (PF) of non-junctional sarcolemma. The intramembranous particles (~8nm) are distributed uniformly over the entire fractured face.

Fig. 1B. Shows a fractured face (PF) of the sarcolemma from a lumbrical muscle of rat denervated for 2 weeks. A large number of particles are apparent in the denervated muscle which are not discerned in the normal (innervated) muscle. These particles are bigger (~15nm) than those seen on this face in the normal muscle and it is likely that they represent extrajunctional acetylcholine receptors in denervated muscle. The inset is a PF of the denervated EDL muscle showing an aggregate with a number of 15nm particles. Arrow in lower left corner indicates direction of shadowing.

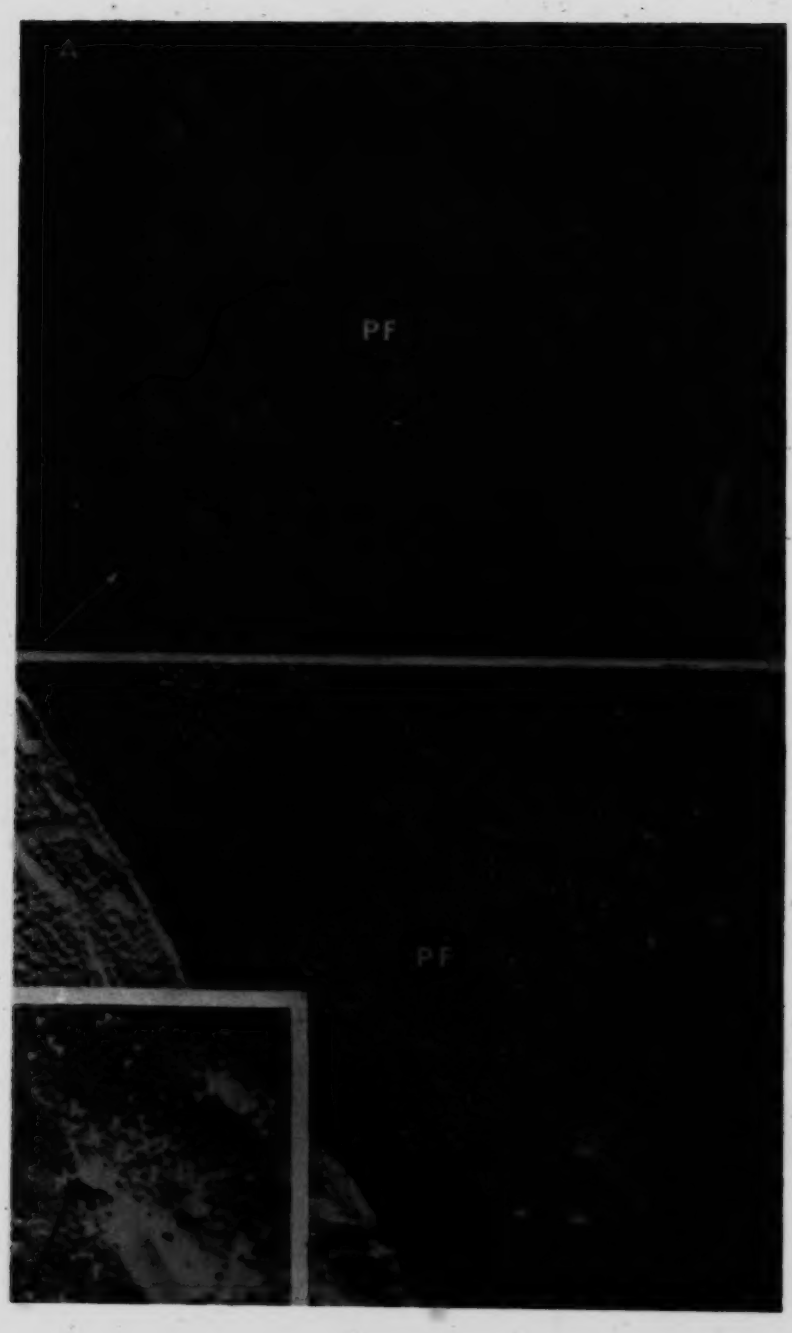

PF
PF

Fig. 2. A comparison of the specific incorporation of the labelled toxin in denervated vs innervated muscle.
1µm thick sections of denervated muscle showing synaptic region with high density of grains (A) and non-synaptic regions with uniformly dispersed grains (C) and clusters of grains (B). Sections of innervated muscle (D) or d-tubocurarine treated denervated muscle (E) do not show grains.
The calibration line in C applies to all the illustrations in this figure.

Fig. 3A. Time-course binding of ^{125}I-α-BGT to crude sarcolemmal preparations from innervated and denervated muscles. The membranes from innervated and denervated muscles were incubated in ^{125}I-α-BGT. The incubation medium contained oxygenated Kreb's ringer, 50 µg/ml membrane protein and ^{125}I-α-BGT (2×10^{-9} M). The membranes were filtered through DE81 cellulose according to the method of Klett *et al.* (*16*). The filter paper was dried and the radioactivity counted in a gamma counter. The specific activity of ^{125}I-α-BGT was 2.192×10^{5} Ci/M.

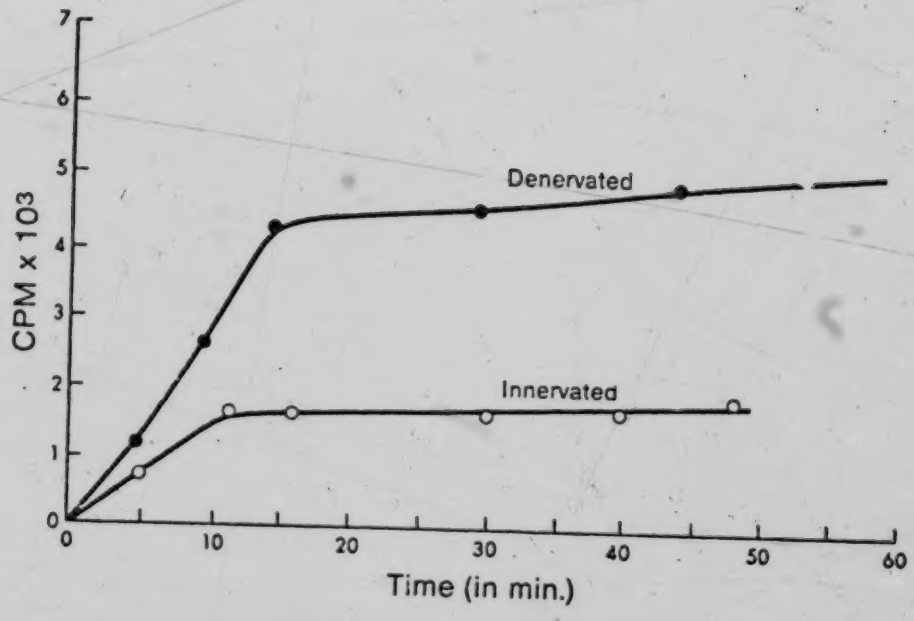

CPM x 103
0
1
2
3
4
5
6
7
Denervated
Innervated
0
10
20
30
40
50
60
Time (in min.)

Fig. 3B. Specific binding of ^{125}I-α-BGT to denervated crude sarcolemmal preparation.

The membrane preparation (50 µg/ml of protein) was incubated in oxygenated Kreb's ringer containing ^{125}I-α-BGT (2×10^{-9} M) for 1 hour at room temperature. The membranes were filtered through DE81 cellulose according to the method of Klett *et al.* (*16*). The filter was dried and the radioactivity counted in a gamma counter. For non-specific binding, the aliquots were incubated in medium containing α-BGT (0.1 mg/ml) for 1 hour and then in labelled α-BGT for 1 hour. The specific activity of ^{125}I-α-BGT was 2.192×10^5 Ci/M.

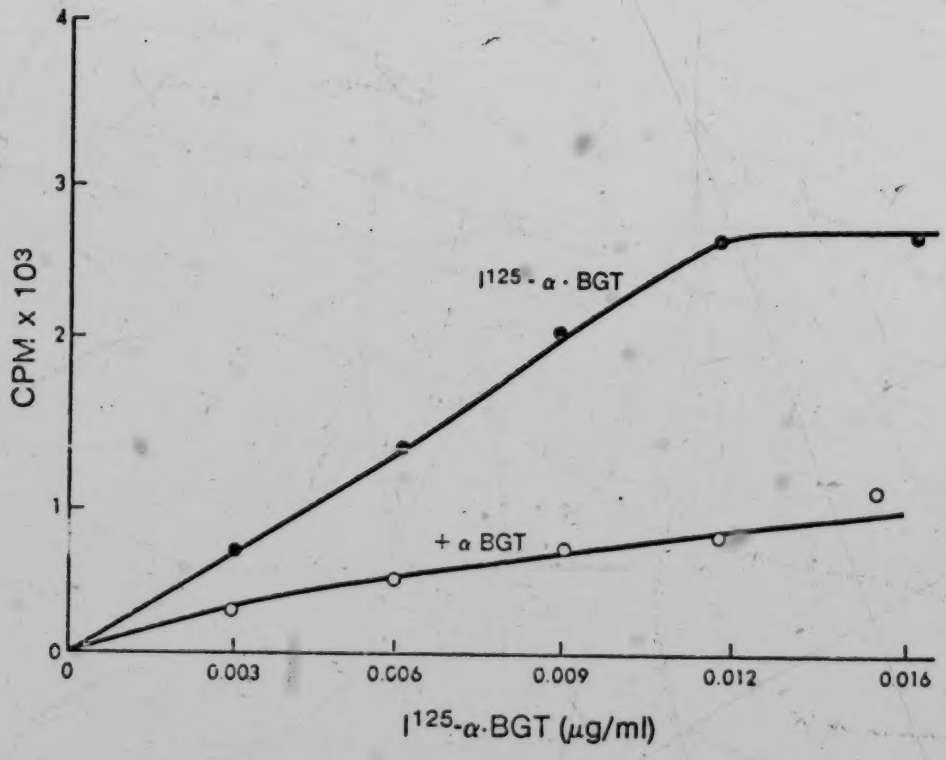

CPM x 103
4
3
2
1
0
I^{125} - α · BGT
+ α BGT
0
0.003
0.006
0.009
0.012
0.016
I^{125}-α·BGT (μg/ml)

Fig. 4. Thin sections from a crude membrane fraction of denervated skeletal muscle incubated in Kreb's ringer containing 0.6 µg/ml of Ft-α-BGT conjugate showing ferritin binding. Several membrane vesicles are seen labelled. Arrows indicate clusters of ferritin particles. Such regions have not been considered in the estimation of Ft-α-BGT binding sites. B is a control from d-tubocurarine incubated material showing paucity of ferritin binding.

A

Fig. 5A. Freeze-etch replica of homogenate from denervated muscle incubated in Kreb's ringer containing 0.6 µg/ml of Ft-α-BGT conjugate showing bumpy appearance of the etched face (ES), presumably due to binding to the conjugate.

Figs. 5B. & 5C. Controls without incubation in conjugate showing relatively smooth etched face (ES). Arrow in lower right corner indicates direction of shadowing.

Table 1

^{125}I-α-BGT counts per min/mg of muscle			
Experiment	^{125}I-α-BGT	d-tubocurarine & ^{125}I-α-BGT	Specific Labelling
Innervated	604	292	312
Denervated	2394	1186	1208

The EDL muscles from innervated and denervated rats were incubated in oxygenated Kreb's ringer with 2×10^{-7} M of ^{125}I-α-BGT for 2 hours. For determining the specificity of binding, another set of muscles was incubated with d-tubocurarine (10^{-4} M) for 1 hour and subsequently incubated with ^{125}I-α-BGT for 2 hours.

Table 2

Experiment	Number of silver grains/μm^2	
	^{125}I-α-BGT	d-tubocurarine & ^{125}I-α-BGT
Innervated	0.6	0.5
Denervated	5	1.4

Distribution of autoradiographic grains in non-synaptic region over 1 μm thick sections of innervated and denervated muscles labelled with ^{125}I-α-BGT. Control in both innervated and denervated muscles represents the preincubation of muscles in d-tubocurarine followed by incubation in ^{125}I-α-BGT. The muscles were incubated under the same condition as mentioned in Table 1.

Table 3A

Counts of Membrane Vesicles (Denervated) Labelled with Ft-α-BGT

Experiment No.	Total Vesicles Counted	Labelled Vesicles	Unlabelled Vesicles
1	31	25	6
2	146	82	64
3	115	70	45
4	99	52	47
	391	229	162

Crude membrane fractions (~50 µg protein) were incubated in Kreb's ringer containing Ft-α-BGT conjugate (0.6 µg/ml). The membranes were filtered through millipore and subsequent to washing, were processed for electron microscopy and embedded in Araldite. The vesicles lying above the filter were randomly counted in thin sections. It is apparent that nearly 60% of the vesicles were labelled. The number of ferritin molecules on the labelled vesicles may vary from 2 or more. The presence of ferritin molecules in the background is rare as the millipore filters were soaked in 2% albumin to minimize non-specific binding (*17*).

Table 3B

Counts of Membrane Vesicles (Denervated) Incubated With d-tubocurarine Followed by Incubation in Ft-α-BGT.

Experiment No.	Total Vesicles Counted	Labelled Vesicles	Unlabelled Vesicles
1	95	15	80
2	153	18	135
3	252	11	241
4	116	23	93
	616	67	549

Counts of membrane vesicles (denervated) incubated with d-tubocurarine followed by incubation in Ft-α-BGT.

The procedure in these experiments was similar to the one outlined in Table 3 except in these experiments, the membrane fractions were incubated in d-tubocurarine (10^{-4} M) prior to incubation in Ft-α-BGT conjugate. The counting of more than 600 vesicles indicate that only 11% of these were labelled.

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CHAPTER IV

Autoradiographic Studies of α-Bungarotoxin Binding Sites (Acetylcholine Receptors) in Innervated and Denervated Skeletal Muscle of the Rat

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J. Neurobiology, submitted

ABSTRACT

A quantitative distribution of α-bungarotoxin (α-BGT) binding sites (Acetylcholine receptor - AchR) has been investigated and correlated with the distribution of intramembranous particles (∿15ηm) observed on fractured face (P-face) of the non-synaptic sarcolemma of denervated muscle. By both light and electron microscope autoradiography, randomly distributed and clusters of α-BGT sites are visualised in denervated muscle. Such distributions of the toxin binding sites corresponds with that of the 15ηm particles on the P-face of denervated muscle. Quantitative studies suggest that upon denervation the toxin binding sites increase 63 fold in non-synaptic sarcolemma. However the density of these α-BGT sites is 4-5 times more than the density of 15ηm particles. These results suggest that each AchR molecule has more than one toxin binding site or that each 15ηm particle is composed of several AchR complexes. In addition to the increase in toxin binding sites on the non-synaptic sarcolemma, notable increase in the number of silver grains is observed in the peripheral sarcoplasm and is speculated to be part of the intracellular pool of receptors. These results are discussed in the light of known biochemical and physiological data on AchR in denervated muscle.

INTRODUCTION

We have previously reported that the presence of ∿15ηm (15-18ηm) intramembranous particles on the P-face is a characteristic feature of the non-synaptic sarcolemma of denervated muscle (Tipnis and Malhotra, 1976, Malhotra and Tipnis, 1978). These particles are distributed singly or occur in clusters. Their density is approximately 400 particles/μm^2. The corresponding fracture face of innervated muscle is devoid of these 15ηm intramembranous particles. Furthermore, the appearance of these particles following denervation coincides with the development of extrajunctional acetylcholine (Ach) sensitivity (Hartzell and Fambrough, 1972). On this basis, we have suggested that these ∿15ηm intramembranous particles are related to the acetylcholine receptors (AchR) (Tipnis and Malhotra, 1976). More recent studies (Tipnis and Malhotra, 1979a, b, c) using ferritin-α-bungarotoxin (α-BGT binds specifically and irreversibly to the Ach receptor) in conjunction with freeze-etching have provided evidence to support our earlier suggestion that the distribution of α-BGT binding sites (AchR) corresponds to the distribution of 15ηm particles. The present study was undertaken to quantitate the α-BGT binding sites in the non-synaptic region of denervated extensor digitorum longus (EDL) and correlate the density of the α-BGT-sites with the reported density of ∿15ηm (15-18ηm) intramembranous particles. Furthermore an attempt is made to study the intracellular distribution of ^{125}I-α-BGT in innervated (control) and denervated muscle cell.

MATERIAL AND METHODS

The female rats of Sprague Dawley strain were used for the present study. The animals were anaesthetized by intraperitoneal injection of 1 ml of 5 mg/ml of sodium pentobarbital (approximately 30 mg/kg) followed by transection of the sciatic nerve in the upper thigh region. The denervated animals were sacrificed 2 weeks after transection as this period of denervation corresponds to the development of optimum extrajunctional sensitivity to Ach (Hartzell and Fambrough, 1972). This period also corresponds to the emergence of 15nm intramembranous particles in the non-synaptic sarcolemma (Tipnis and Malhotra, 1976).

Preparation of I^{125}-α-BGT

Pure α-BGT obtained from Miami Serpentarium was iodinated by Radiopharmacy Center, University of Alberta. The specific activity and the protein concentration of iodinated α-BGT were 207.2 Ci/mM and 3.6 mg/ml respectively.

Incubation of Excised Muscles in ^{125}I-α-BGT

The animals were sacrificed 15 days after transection of the nerve and the extensor digitorum longus (EDL) muscle from the denervated as well as control group were individually tied at both ends to a wooden stick and transferred to Kreb's Ringer. A mixture of 95% O_2 and 5% CO_2 was continuously bubbled through the Ringer solution maintained at 37°C and the muscles were incubated as follows: One set each from control and denervated group was incubated in Kreb's Ringer containing ^{125}I-α-BGT (2 μg/ml) for a period of 2 h.

Another set of muscle was first incubated for 1 h in Kreb's Ringer containing d-tubocurarine (10^{-4} M) followed by incubation in Ringer containing labelled toxin (2 µg/ml) for 2 h. The muscles after extensive washing were fixed in 2% glutaraldehyde and after removal from the wooden sticks, the radioactivity was counted in a gamma counter (Baird Atomic). The muscles after post-fixation in 2% OsO_4 in 0.1 M phosphate buffer (pH 7.0) were dehydrated in graded series of ethanol and embedded in Araldite.

Light Microscope Autoradiography

1 µm thick sections cut from the tissue blocks embedded in Araldite were transferred onto clean glass slides. The slides were coated with Ilford L_4 emulsion (diluted 1:1) at 50°C and stored at 0°C. The slides after exposure for 3-7 days were developed in Microdol X (at full strength) for 3 minutes and after fixing in 25% sodium thiosulphate were stained with 2% Phenylenediamine and examined by phase contrast microscopy.

Electron Microscope Autoradiography

Pale gold sections prepared from the synaptic and non-synaptic regions of the muscle were transferred to copper grids. The sections were stained with 2% uranyl acetate and lead citrate. The measurements of section thickness were done with a Philips EM 300 electron microstope according to the method given by Weibull in Philips manual. The magnification was set at Step 11 and the beam current was adjusted to 80 at the exposure meter. The grid was introduced into the microscope and the section of the tissue was positioned under the electron beam and the reading R of exposure meter was recorded.

The percentage of scattered electrons was calculated from the following formula:

$$P = 100 \times \frac{80 - R}{80}$$

The thickness of section was extrapolated from percentage of the value of scattered electrons in the graph provided by the author in Philips manual. The grids were transferred on a formvar film which was picked up on glass slide. The grids were coated with carbon and then with Ilford L4 emulsion. After exposure for 4-5 weeks, the slides were developed as described for light microscope autoradiography.

The autoradiographs were examined in a Philips EM300 electron microscope. The grids from different blocks were chosen at random and neuromuscular junctions were photographed and printed at a magnification of 30,000X. The regions of non-synaptic sarcolemma were photographed and enlarged to 6600X. The visual counting of grains was done on the viewing screen of the electron microscope and the location of the silver grain with respect to various organelles was recorded.

(a) Localisation of Radioactive Source (see Appendix IV)

This was analysed according to the method of Fertuck and Salpeter (1976) which is briefly given below: 1) A plastic lamina with series of circles is prepared. 2) Lamina is placed over the grain and the center of the smallest circle circumscribing the grain was punctured with a needle. 3) The distance from the midpoint of the grain perpendicular to the line drawn along the top of synaptic sarcolemma was measured. In order to determine the grain density (grains/unit area) area was first measured by superimposing

a lattice with uniformly spaced points on the circle circumscribing the silver grain. The lattice points were punctured with a needle and the distance from each of these lattice points to the line was measured. Dividing the grain count in each column by the corresponding set of points gives the grains/μm^2. In this tabulation, the grains within 30 mm (on the micrographs) on axonal as well as on muscle side were counted. A total of 373 grains and 3320 points were considered in the quantitation. The distance is expressed in units of Half-distance (HD is defined by Salpeter *et al.* (1969) as the distance from the hot line source within which half the developed grains fall. In the present investigation HD value determined from integrated grain distribution is 100nm). The grain density is normalized by setting the grain count containing the source (HD=0) to unity. The other values of the grain count are adjusted proportionately and plotted against distance in units of HD.

(b) Quantitation of α-bungarotoxin Sites (see Appendix IV)

In this investigation, the grain density (grains/unit) was determined according to Fertuck and Salpeter (1976).

$$\text{Sites}/\mu m^2 = \frac{G \times d}{124{,}800\ (1-e^{-0.01155t})} \times \frac{A}{S_o C}$$

G = grain density

d = inverse of decays needed for one developed grain.

The sensitivity value was determined from sections according to the procedure given by Williamson (1977) So = specific activity

of ^{125}I-α-BGT sites at the time of exposure period (expressed in Ci/mM) . C = disintegrations/minute = 2.22×10^{12} A = Avogadro's number = 6.023×10^{20} molecules/mM. In the present study all grains within 3HD on either side of membrane were counted. In quantitation of α-BGT sites, 300-500 grains were counted. Muscle background was measured at random from different regions of the fibers and binding sites due to background were calculated.

RESULTS

Light Microscope Autoradiography

We have previously shown that the binding of I^{125}α-BGT to muscle is specific and is inhibited (50%) by d-tubocurarine (Tipnis and Malhotra, 1979c). Autoradiographs prepared from the toxin incubated as well as control material show that in innervated muscle, high density of silver grains is localised in the synaptic region (Fig. 5) whereas the non-synaptic sarcolemma, shows only a few grains (Fig. 1). In denervated muscle, the non-synaptic sarcolemma shows increased density of grains, dispersed at random (Fig. 2) or present in aggregates (Fig. 3). The non-synaptic sarcolemma of denervated muscle incubated in d-tubocurarine is essentially devoid of grains (Fig. 4), thereby indicating the specificity of labelling. The apparent density of silver grains in clusters in identifiable non-synaptic sarcolemma of denervated muscle (Fig. 3) is much less than that in the region of the synapse (Fig. 5).

Electron Microscope Autoradiography

(a) Identification of Radioactive Source.

Although the myoneural junctions exhibit much higher number of

silver grains (Fig. 6) than elsewhere in the muscle, it is essential to determine the degree of radiation spread from the synaptic sarcolemma.

The normalized grain distribution relative to post-junctional membranes is given in Fig. 7. The highest density is located near the source and falls on either side of source with increasing HD distance. The histogram approximates the theoretical grain curve of a line source (Salpeter *et al.*, 1969) as well as experimental curve for neuromuscular junction (Fertuck and Salpeter, 1976). In our experiment, 65% of the total grains are located within the distance of 3HD. Salpeter *et al.* (1977) have reported that 90% of the silver grains due to ^{125}I source fall within a HD value of 3.5 (280ηm). Therefore in the present study, the grains within 3HD (300ηm) are considered to be due to the labelling of the receptor at the synaptic sarcolemma.

(b) Estimation of α-BGT Binding Sites in Innervated and Denervated Muscle.

A neuromuscular junction labelled with ^{125}I-α-BGT is shown in Fig. 6. The α-BGT binding sites estimated relative to sarcolemmal membrane (synaptic and non-synaptic) are given in Table I. The density of these sites is much higher in synaptic region than that in the non-synaptic region of innervated muscle. Upon denervation there is approximately 50 fold increase in binding sites in the non-synaptic sarcolemma of denervated muscle. In most of these areas, the grains are distributed at random (Fig. 8 and 9) although occasionally

patches with high density of silver grains are encountered (Fig. 10). Based on examination of several grids, it appears that these regions of high density of silver grains correspond to the hot spots of AchR (Fischbach and Cohen, 1973; Ko *et al.*, 1977). Their distribution also corresponds with that of the clusters of 15nm intramembranous particles seen in freeze-fractured non-synaptic sarcolemma of denervated muscle (Fig. 11 and 12).

A distribution of silver grains lying directly over the muscle organelles is given in Table II. It may be noted that the number of silver grains lying over non-synaptic sarcolemma of denervated muscle is much higher than that in the innervated muscle. Although the number of silver grains in sarcoplasmic reticulum, mitochondria and nucleus does not differ markedly in innervated and denervated muscle, it is of interest that the increase in silver grains is observed in perinuclear and subsarcolemmal sarcoplasm of denervated muscle. It is possible that such an increase represents the intracellular pool of AchR.

Freeze-fractured Faces of Non-synaptic Sarcolemma of Innervated and Denervated Muscle.

The results of this study have been described previously (Tipnis and Malhotra, 1976, Malhotra and Tipnis, 1978) and therefore only the salient features relevant to the present investigation are mentioned here.

The non-synaptic sarcolemma of the skeletal muscle is markedly altered after denervation. The most conspicuous change is in

the appearance of ∿15ηm (15-18ηm) intramembranous particles on the protoplasmic fractured face (P-face) of the non-synaptic sarcolemma. These particles are distributed singly (Fig. 11) in random manner or in clusters of 10-50 intramembranous particles per cluster (Fig. 12). The density of these randomly dispersed particles is 400/μm^2. The corresponding face (P-face) of non-synaptic sarcolemma of innervated muscle has 8ηm intramembranous particles randomly dispersed at a density of 2000/μm^2, but the 15ηm particles are lacking from this face of the innervated muscle.

DISCUSSION

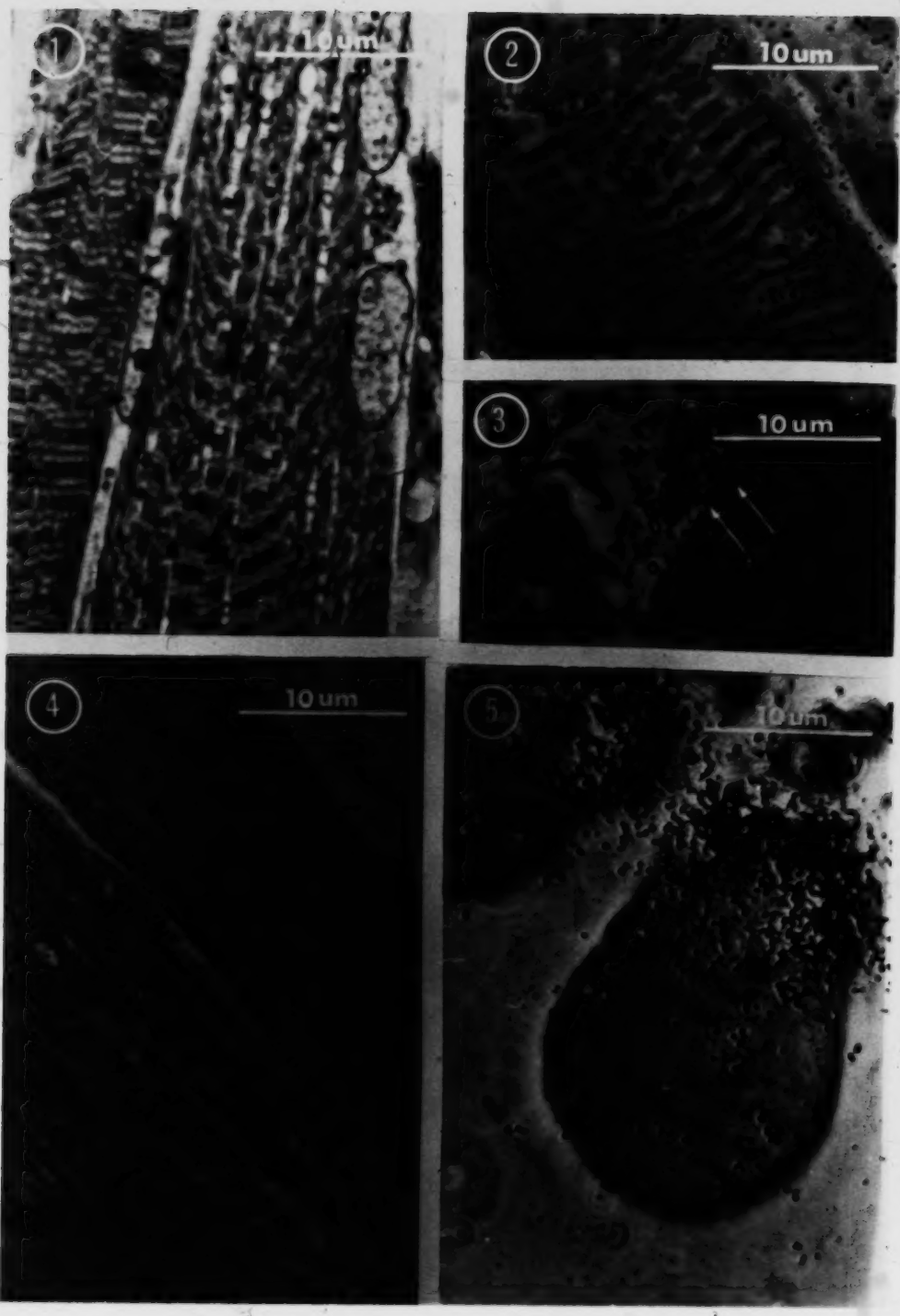
The major contribution of the present study is correlation of light and electron microscope autoradiographic data with the one reported from freeze-fracture studies (Tipnis and Malhotra, 1979c). The freeze-fracture studies indicate two types of distributions of ∿15ηm intramembranous particles on the P-face of non-synaptic sarcolemma of denervated muscle. Similar spatial distributions of silver grains from ^{125}I-α-BGT labelling of denervated muscle are noted at the level of light and electron-microscope. The evidence that the 15ηm particles are component of AchR has been recently provided by freeze-etch studies of ferritin-α-BGT labelled sarcolemma of denervated muscle (Tipnis and Malhotra, 1979c). Two types of spatial distributions of the ferritin-α-BGT binding sites are discernible corresponding to the two distributions of 15ηm intramembranous particles, and to the two types of distributions of silver grains (^{125}I-α-BGT). Its known that the sensitivity to Ach in extrajunctional sarcolemma of non-innervated muscle is not uniform

but exhibits patches of high sensitivity (hot spots) (Fishbach and Cohen, 1973). The clusters of α-BGT binding sites correspond to the hot spots of high Ach sensitivity. There does not appear to be a 1:1 relationships between the estimated number of α-BGT binding sites and the number of intramembranous particles (i.e. 400/μm^2). The reasons may be that each intramembranous particle is a composite of more than one AchR complex, or that each receptor molecule may bind two or more toxin molecules.

It is of interest that there is an increase in the number of silver grains in the subsarcolemmal and perinuclear sarcoplasm which has numerous Golgi vesicles. We have previously reported that in denervated muscle there is a considerable increase in endoplasmic reticulum and ribosomes in subsarcolemmal region (Tipnis and Malhotra, 1977). Therefore, the observed increase in number of silver grains in perinuclear and subsarcolemmal sarcoplasm leads us to suggest that it represents a part of intracellular pool of receptors. Studies related to AchR in cultured muscles of chick indicate that incorporation of AchR into plasma membrane occurs 2-3 h after their entry into the intracellular pool (Fambrough and Devreotes, 1978). The inhibitors of protein synthesis (e.g. Puromycin) block the appearance of new receptors on the surface of the membrane only after a lag period of 3 h. The newly synthesized AchR sites are now reported to be in Golgi apparatus where they are inaccessible to the ligand action. However, if the membrane is made permeable to the ligand molecule, the receptor sites (that constitute 28% of surface receptors) could be labelled (Fambrough and Devreotes, 1978). It is possible that the observed increase in the silver grains in peripheral and

small percentage of total intracellular receptors. Furthermore, though in the present study detergents were not used to make membranes permeable to toxin, it is conceivable that they became permeable by the trauma caused during incubation resulting in the labelling of intracellular receptors with ^{125}I-α-BGT.

Figs. 1-5. Autoradiographic localization of α-BGT binding sites (AchR) in 1um thick sections. The innervated muscle showing paucity of silver grains in non-synaptic sarcolemma (Fig. 1). Denervated muscle shows randomly distributed (Fig. 2) and clusters (Fig. 3) of silver grains in non-synaptic region. Control sections of muscle pretreated with d-tubocurarine do not show silver grains (Fig. 4). The neuromuscular junction muscle shows much higher concentration of grains (Fig. 5) than that seen in the non-synaptic region (denervated muscle) with clustered silver grains (Fig. 3).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5
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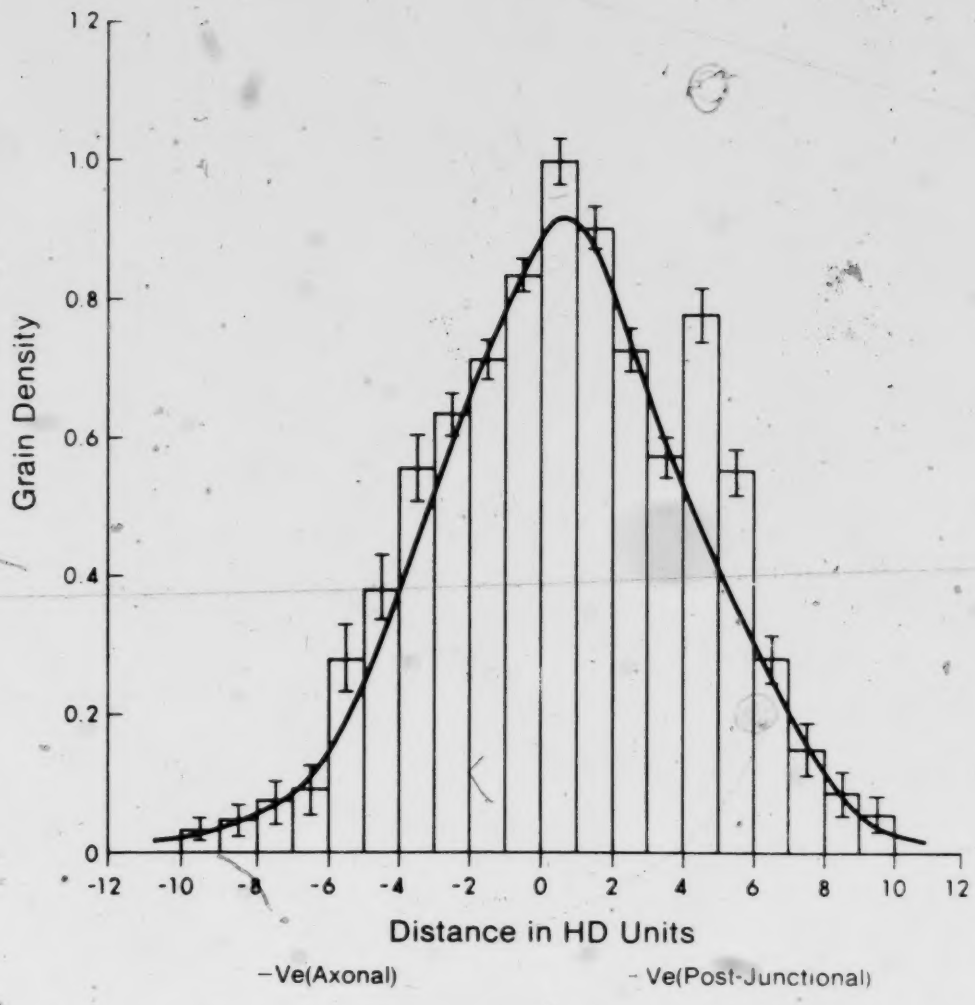
Fig. 6. Electron microscope autoradiograph of innervated muscle incubated in ^{125}I-α-BGT showing the localisation of toxin binding sites (AchR, seen as silver grains) in the synaptic region. The details of incubation and subsequent preparation are given in Material and Methods.

Fig. 7. Histogram of grain density distribution of silver grains in autoradiographs of neuromuscular junction of muscle incubated with ^{125}I-α-bungarotoxin. The origin (0) represents the line drawn along the post-junctional membrane. The distance from the source (0) is measured in units of HD (100nm). The grain density at the source was set at unity and other values are adjusted proportionately. The normalized grain density is plotted against distance in units of HD. The smooth curve corresponds to the expected grain distribution for the line source (Salpeter *et al.*, 1977).


Grain Density
1.2
1.0
0.8
0.6
0.4
0.2
0
-12
-10
-8
-6
-4
-2
0
2
4
6
8
10
12
Distance in HD Units
–Ve(Axonal)
– Ve(Post-Junctional)

Figs. 8-10. Electron microscope autoradiograph of non-synaptic sarcolemma of denervated muscle incubated with ^{125}I-α-BGT. The silver grains (α-BGT sites, arrows) are seen as randomly distributed (Figs. 8 and 9) and as clusters (Fig. 10). Fig. 8 also shows silver grains in the sarcoplasm. The incubation and subsequent processing of the muscle are given in Material and Methods.

Figs. 11-12. Freeze-fractured replicas from non-synaptic sarcolemma of Extensor digitorum longus muscle showing the presence of 8ηm intramembranous particles on P-face of innervated muscle (Fig. 11). Fig. 12 shows a comparable fractured face of the denervated muscle: 15ηm particles distributed singly (small arrows) or in clusters (circles, and inset) are apparent. Such particles are lacking from the corresponding face of the innervated muscle (Fig. 11). Arrow in the lower right corner indicates direction of shadowing.

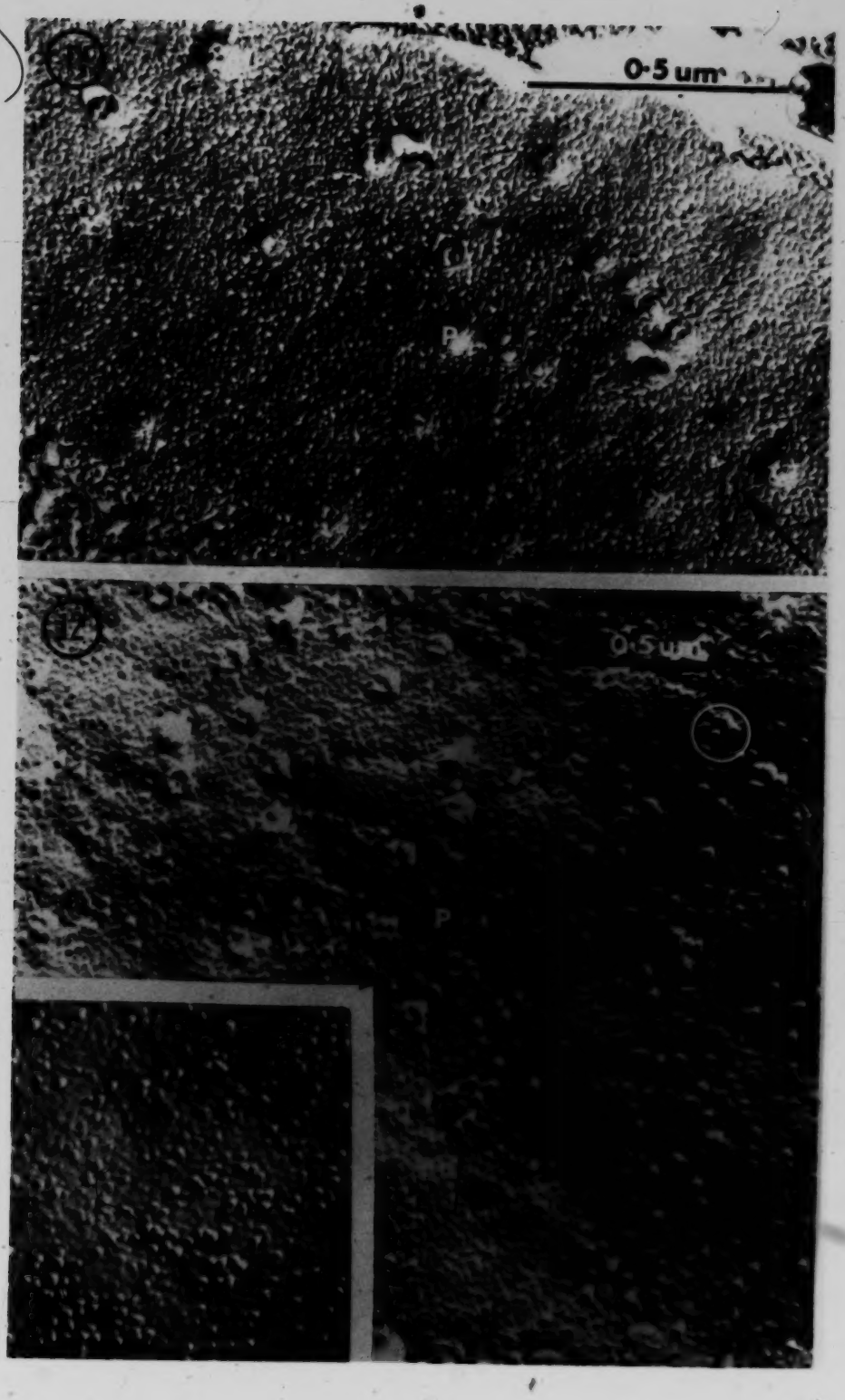

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Table I

α-bungarotoxin binding sites/μm^2 relative to sarcolemmal membrane of innervated and denervated muscle

Experiment	Region of Muscle	Binding sites/μm^2
Innervated	Synaptic sarcolemma	14,000
	Non-synaptic sarcolemma	25
	Muscle background	174
Denervated	Non-synaptic sarcolemma	1590

The electron microscope autoradiographs from synaptic and non-synaptic regions of muscle were used in the above quantitation. The grain density/μm^2 and its conversion to ^{125}I-α-BGT sites/μm^2 was done according to the method of Fertuck and Salpeter (1976). The non-synaptic sarcolemma after denervation shows more than 60 fold increase in α-BGT sites. Approximately 400 grains were counted for each of the above estimates.

Table II

Counts of silver grains in muscle labelled with ^{125}I-α-BGT

	Experiment	
	Innervated	Denervated
Total No. of Grains	611	1305
Non-synaptic Sarcolemma	54	674
Sarcoplasmic reticulum	185	236
Myofibrils	151	148
Subsarcolemmal Sarcoplasm	15	111
Perinuclear Sarcoplasm	4	22
Nucleus	18	25
Lysosomes	10	25
Mitochondria	74	60

The grids were processed for electron microscope autoradiography as given in Material and Methods. The silver grains lying directly over muscle organelles were counted on the viewing screen of the electron microscope. The above data show that the number of grains lying over non-synaptic sarcolemma, perinuclear sarcoplasm and subsarcolemmal sarcoplasm increase upon denervation of the muscle.

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CHAPTER V

Junctional and Extrajunctional Acetylcholine Receptors

(U. R. Tipnis and S. K. Malhotra)

Can. J. Physiol. Pharmacol., in press

Introduction

Acetylcholine receptors (AchR) are being extensively studied because their functioning is of interest to interdisciplinary scientists, such as neurobiologists, biochemists, immunologists, pharmacologists, and cell and developmental biologists (Robertson 1979). Apart from their well-known function as a receptor for acetylcholine (Ach), their possible regulatory role in the growth of the presynaptic terminal (Freeman 1977) and their involvement in the pathological state characterized as myasthenia gravis (Kao and Drachman 1977; Heinemann *et al.* 1978) are under investigation. AchR are most abundant at the electroplaques of electric fish, which provide a convenient and rich source for biochemical and structural studies (see The Synapse, Cold Spring Harbor Symposium, 1976, Vol. 40; Heidmann and Changeux 1978). In recent years AchR from neuromuscular junctions of vertebrates have also provided a good deal of data (see Edwards 1979; Fambrough 1979). Besides, the AchR in embryonic muscles (Steinbach *et al.* 1979) and the extrajunctional AchR that are produced following denervation of adult skeletal muscle (Miledi and Potter 1971; Tipnis and Malhotra 1976) are also of current interest

(in particular, their role(s) and comparison with the junctional AchR). The present article, then, primarily deals with the junctional AchR and their comparison with the extrajunctional AchR in vertebrates.

Dale (1914) classified the AchR into nicotinic and muscarinic type, depending on their sensitivity towards nicotine and muscarine. The activation of these two receptors by a common ligand, Ach, is due to the binding of the receptors to two different portions of the Ach molecule. The carbonyl side of the Ach molecule reacts with the nicotinic receptor, whereas the methyl side reacts with the muscarinic receptor (Chothia 1970). The introduction of various other pharmacological agents, agonists and antagonists, has further confirmed the above two main categories of AchR. For example, agonists like tetramethylammonium (TMA), dimethylphenyl piperazinium (DMPP), phenyltrimethylammonium (PTMA) and suxamethonium stimulate nicotinic receptors, whereas agents like acetyl B-methylcholine, oxotremorine, pilocarpine and arecoline stimulate the muscarinic receptor (see Goodman and Gilman 1970). Nicotinic receptors are blocked by curare, whereas muscarinic receptors are blocked by atropine (see Goodman and Gilman 1970).

The AchR are widely distributed amongst invertebrates (Kehoe

1972; Kehoe *et al.* 1976; Dudai 1979; Ross and Triggle 1972) and vertebrates (Fambrough 1979; Vogel and Nirneberg 1976; Krnjevic 1974). However, the electrogenic cells and their homologous skeletal muscle cells have been more thoroughly investigated in respect of structure, characterization and biosynthesis of AchR. The AchR from both these cells are of nicotinic type (see Karlin *et al.* 1976). The studies devoted towards physical and biochemical characterization have been facilitated by the availability of specific probe α-bungarotoxin (BGT) and related neurotoxins. These toxins are obtained by fractionation of crude snake venom from various species of land and sea snakes (see Lee 1979). These toxins can be conjugated to ^{125}I-, ^{3}H-, horseradish-peroxidase (Jacob and Lentz 1979) or ferritin (Hourani *et al.* 1974; Tipnis and Malhotra 1979a, b, c) for correlative biochemical and structural studies. Immunochemical techniques are also being applied for structural studies (Klymkowsky and Stroud 1979) as well as for cross reactivity between subunits of AchR (Lindstrom 1976; Claudio and Raftery 1977).

Acetylcholine Receptor Complex

AchR is a glycoprotein as the receptor binds concanavalin A and other lectins (Meunier *et al.* 1974; Brockes and Hall 1957a; Almon and Appel 1976b; Boulter and Patrick 1977; Patrick *et al.* 1977; Fambrough 1979). The AchR is a transmembrane protein and is exposed on both surfaces of the plasma membrane (Heidmann and Changeux 1978; Fambrough 1979). The evidence for the transmembrane nature of this protein has come from a variety of experimental studies: (1) X-ray diffraction studies on *Torpedo californica* electroplaques reveal that the receptor extends by 5.5 nm on the outside and by 1.5 nm on the inside (Ross *et al.* 1977). The extension of AchR beyond the bilayer has been further demonstrated by labelling the membranes of *Torpedo* with rabbit and goat anti-receptor antibodies coupled directly or indirectly to colloidal gold or to ferritin (Klymkowsky and Stroud 1979). (2) Immunochemical studies in which the receptor rich microsacs prepared from *Torpedo* and labelled with ferritin-conjugated antibodies to AchR have demonstrated the binding of the conjugate to the receptor protein on both sides of the membrane (Tarrab-Hazdai *et al.* 1978). (3) Huang (1979) has reported that the membranes prepared from electroplaques of *Torpedo* are a mixture of inside-out as well as right-side-out vesi-

cles (1:1). In the right-side-out vesicles the receptor sites, when occupied with ^{125}I- BGT, are resistant to chymotrypsin. The radioactive toxin-receptor complex prepared from these membranes by solubilization with Triton X-100 has the same sedimentation constant as that prepared from membranes without chymotrypsin digestion, i.e., ∿13 S. If the membrane vesicles are first labelled with cold BGT and then treated with -chymotrypsin, the resulting preparation, when treated with Triton X-100, provides a AchR complex that could be labelled with ^{125}I- BGT. However, this radioactive receptor complex has a sedimentation constant of ∿7 S, thereby indicating that chymotrypsin had digested a part of the receptor molecule. This part must belong to the inside-out vesicles and the receptor molecule is accessible to chymotrypsin.

The morphological studies devoted towards understanding the structure-function relationship of the receptor protein have been carried out on cholinergic-rich synapses like electric organ (Changeux *et al*. 1976; Cartaud *et al*. 1978; Klymkowsky and Stroud 1979) and neuromuscular junction of frog (Peper *et al*. 1974) and rat (Rash and Ellisman 1974). The negatively stained preparations of receptor-rich membranes from *Torpedo* show the presence of parti-

cles 8-9 nm in diameter and arranged in rosettes (Cartaud *et al.* 1978; Klymkowsky and Stroud 1979). Each rosette has a central pit of 1.5 nm and is composed of 3-6 subunits (Cartaud *et al.* 1978). Corroborative evidence for the subunit structure is further provided by employing freeze-fracturing in which the fracturing of the membrane occurs in the hydrophobic region, thereby revealing the membrane interior. When the membranes are deeply etched the surface of the membrane can be visualized as well. Such studies on membranes of electric organ of *Torpedo* have demonstrated the presence of a high density of intramembranous particles on the inner half (P-face) of the plasma membrane. These particles are 8-9 nm in diameter and occur in rosettes similar to the ones observed in negatively stained preparations. Deep etching of the membrane enables visualization of a similar subunit structure of the receptor on the surface of the membrane as that seen on the fractured face and in negatively stained preparations (Cartaud *et al.* 1978).

The freeze-cleaved replicas of mammalian neuromuscular junctions reveal 2-3 rows of 11-15 nm particles on the P-face near the juxtaneural portions of the postsynaptic sarcolemma. The density of these particles is 1700-2000/μm^2 (Rash and Ellisman 1974; Malhotra and Tipnis 1978). The attempts to correlate the intra-

membranous particles viewed in freeze-cleaved replicas with a specific protein has been a difficult and challenging problem. The suggestion that the particles observed on postsynaptic sarcolemma are the AchR protein has received support from the following indirect observations. (1) The electron micrographs of the motor endplate show a thickening of the postsynaptic membrane near the juxtaneural portion of the fold. [Since the AchR protein and acetylcholinesterase are the major postsynaptic membrane proteins, Birks *et al.* (1960) have suggested that membrane thickenings may be due to protein molecules.] (2) The quantitation of AchR labelled with ^{125}I-BGT indicates that the receptor is more highly concentrated at the top of the junctional folds near the synaptic cleft (Fertuck and Salpeter 1976). (3) The density of intramembranous particles on the P-face of the postjunctional sarcolemma remains unaltered after denervation for a period of 15 days (Malhotra and Tipnis 1978). This is consistent with the observations that BGT binding sites do not suffer a change in number after denervation (for 10 days) of the soleus muscle of rat (Frank *et al.* 1976).

AchR protein represents about 30% of the membrane protein in the receptor-rich fragments of *Torpedo* electric organ (see Heidmann and

Changeux 1978). These receptor-rich membrane fragments, when subjected to SDS gel electrophoresis, yield four components of molecular weights 40,000, 43,000, 50,000 and 60,000 in *T. marmorata* and 39,000, 48,000, 58,000 and 64,000 in *T. californica* (Heidmann and Changeux 1978). In *Electrophorus*, receptors contain two or three polypeptides of molecular weights 40,000, 47,000 and 53,000 (Merlie *et al.* 1978). By using photo-affinity labelling anologues for the AchR of *T. californica*, Witzemann and Raftery (1977) have concluded that BGT binds to the 40,000 M.W. subunit and that this subunit may interact with the other subunits of AchR. Sumikawa and O'Brien (1979) have reported that the smallest subunit of *Torpedo* (38,000 M.W.) and eel (39,000 M.W.) both bind to 4-M (maleimido)-benzyltri[^{3}H] methylammonium iodide [(^{3}H)MBTA] and can cross-react between the two species and is, therefore, considered to contain the Ach binding site. By using ^{3}H-MBTA, Froehner *et al.* (1977) have purified the AchR protein from the denervated rat leg muscle. Mammalian AchR had two binding sites for ^{3}H-MBTA, whereas the *Torpedo* AchR had only one such site. The molecular weights of polypeptides from rat muscle that bind MBTA are 45,000 and 49,000. The binding of MBTA by these polypeptides is blocked

by α-toxin. These results would suggest that there are apparent differences in the AchR complex in fish and mammalian membranes which require further substantiation. Also, purified receptors from embryonic skeletal muscle cells of fetal calf grown in tissue culture contain a single class of polypeptide of 41,000 M.W. (Merlie *et al.* 1978). Furthermore, the question of the detailed structure of the AchR is being debated (see Hider 1979). Based on studies on mammalian denervated muscle and electric organ of *Torpedo*, respectively, Shorr *et al.* (1978) and Changeux *et al.* (1979) have considered the possibility that the AchR is essentially an oligomer of 41,000 M.W. subunits. However, Vandlen *et al.* (1979) have reported that the AchR purified from *Torpedo* electric organ does contain four different polypeptides of molecular weights 40,000, 50,000, 60,000 and 65,000.

Notwithstanding the above findings on polypeptide composition, Lindstrom *et al.* (1978) have immunologically examined the properties of four polypeptide chains isolated from the AchR of the electric organ of *T. californica* and compared them with those of mammalian AchR. There are some determinants in the AchR from human and rat muscle which specifically cross-react with the determinant characteristics of all four polypeptide chains of

AchR from *Torpedo*. Antibodies against the four polypeptide chains of *Torpedo* receptor cross-react *Torpedo* with AchR in rat muscle. These findings are of interest since parts of the AchR have been conserved through evolution. However, it should be pointed out that these polypeptide chains are immunologically distinct and differences have been reported in the pattern of polypeptide chains of receptors of the electric organ of *Torpedo* and eel.

The identification of the functional significance of each of the subunits of the AchR is rather a difficult task. Whereas the role of the smallest subunit in *Torpedo* and *Electrophorus* is the binding of Ach, that of 43,000 M.W. is as yet questionable and that of the remaining larger subunits is not known at all. Sobel *et al.* (1978) have obtained by SDS/polyacrylamide gel electrophoresis two major polypeptides (M.W. 40,000 and 43,000) from the highly purified subsynaptic membrane fragments of *T. marmorata*. The 40,000 subunit binds to 4-(N-maleimido)phenyl[^{3}H]trimethylammonium [(^{3}H)MPTA] and is therefore assumed to be the AchR protein. The properties of the 43,000 subunit are different from those of the 40,000 subunit in the following ways. (1) It does not bind Con A and therefore it is not a glycoprotein. (2) It has glycine, alanine and valine residues not found in the AchR

protein. (3) It makes high molecular aggregates under conditions where the receptor protein (9S) remains in solution. Furthermore, in the presence of quinacrine, both AchR rich membranes and the 43,000 subunit give a fluorescence signal sensitive to histrionicotoxin (HTX). The 40,000 subunit, on the other hand, does not interact with quinacrine and is insensitive to HTX. It was suggested, therefore, that the polypeptides of M.W. 40,000 and 43,000 carry the AchR site and the local anaesthetic site, respectively. The experimental evidence in contradistinction to that of Sobel *et al.* (1978) has been presented by Neubig *et al.* (1979) who have reported on the solubilization of a peptide of M.W. 43,000 from alkaline-extracted postsynaptic membranes of *Torpedo* rich in AchR. The remaining major peptides are of M.W. 41,000, 50,000 and 65,000 which are characteristic of purified AchR. The postsynaptic membranes, after alkaline extraction, retained the properties to bind ^{3}H-α-toxin and a local anaesthetic (eg. dimethisoquin). Such membrane preparations were also functional in the manifestation of ^{22}Na efflux, thereby indicating that the peptide of M.W. 43,000 is distinct from the peptide(s) of the AchR which are involved in the binding of the

AchR and of local anaesthetics. Changeux *et al.* (1979) have also successfully reconstituted AchR rich membranes from *Torpedo* from which M.W. 43,000 polypeptide has been removed by alkaline extraction, thus providing further support that the ionophore and the Ach binding site are carried by the polypeptide of M.W. 40,000.

Distribution of Acetylcholine Receptor

Skeletal Muscle

Based upon electrophysiological (Miledi 1960) and autoradiographic data (Porter *et al.* 1973; Fertuck and Salpeter 1976), the AchR are well recognized to be concentrated in the sarcolemma in the region of the neuromuscular junction (Fig. 1). They have a much higher density at the tips of the junctional folds than at the depths of these folds (Fertuck and Salpeter 1976). There is a wide variation in the number of AchR reported for different muscles (see Edwards 1979), eg. electroplaques of *Electrophorus* have 50,000 receptors/μm^2 (Bourgeois 1976) and estimates for mammalian muscles range from $\sim$8700/μm^2 (Albuquerque *et al.* 1974) to 46,000/μm^2 (Fertuck and Salpeter 1976). This variation may reflect differences in the type of muscle and species or differences in the method of application of BGT used to estimate the receptor number (Albuquerque *et al.* 1974; Fertuck and Salpeter 1976). These estimates are based upon the assumption that there is one toxin binding site per receptor complex, though there are reported to be two such binding sites (Albuquerque *et al.* 1974; Karlin *et al.* 1978). The number

of receptors declines with the distance from the neuromuscular junction (Fertuck and Salpeter 1976). The extrajunctional receptors appear to vary in number depending on the type of muscle studied. In slow muscle the extrajunctional sarcolemma has high and low patches of Ach sensitivity (Miledi and Zelena 1966; Albuquerque and Thesleff 1968; Albuquerque and McIssac 1970) and the number of receptors is estimated to be 22-25 binding sites/μm^2. The fast muscle (like EDL), in which the sensitivity to Ach is restricted around the neuromuscular junction and the regions surrounding it, has only 5-10 extrajunctional binding sites/μm^2 (Hartzell and Fambrough 1972). The density of extrajunctional receptors in embryonic as well as denervated muscle is far higher than in the adult (innervated) muscle (Fambrough 1979).

Denervated Muscle

In contrast to the innervated muscle, the denervated muscle responds to the application of Ach in the extrajunctional region (Axelsson and Thesleff 1959). An increase in chemosensitivity begins four days after denervation and appears to spread over the extrajunctional membrane by seven days (Axelsson and Thesleff 1959). After the denervation of fast muscle the increase in the chemosensitive

zone does not occur centrifugally but first appears near the end-plate and then near the myotendinous region. The mid-portion of the extrajunctional region is the last to acquire chemosensitivity. Soleus, which in innervated state has high Ach sensitivity in the neuromuscular junction and myotendinous junction, exhibits regions of relatively high and low Ach sensitivity in the extrajunctional sarcolemma. This muscle, when denervated, exhibits a uniform increase in Ach sensitivity along the length of the fiber. Also, the appearance of chemosensitivity occurs earlier in soleus than in EDL (Albuquerque and McIssac 1970).

The increase in extrajunctional Ach sensitivity, as shown in denervated rat diaphragm, occurs linearly with the increase in the number of AchR in the extrajunctional sarcolemma (Hartzell and Fambr 1972). Such an appearance of extrajunctional AchR in denervated EDL muscle is shown in Fig. 2, which is an autoradiograph of ^{125}I-α-BGT treated material. It is now commonly accepted that the extrajunctio Ach sensitivity results from the biosynthesis of AchR and their incorporation into the sarcolemma (Hartzell and Fambrough 1973; Brockes and Hall 1975; Devreotes and Fambrough 1976). However, Blunt *et al.* (1975) have considered the possibility that the

extrajunctional AchR in the innervated muscle membranes are somehow masked and inaccessible to the action of drugs. When the muscle is denervated, lytic action of phagocytic cells causes unmasking of the extrajunctional receptors. This suggestion is based upon experimental manipulations in which the appearance of extrajunctional Ach sensitivity could be prevented in both EDL and soleus muscles by the arrest of cell division by vincristine (mitotic inhibitor). Also, the authors further report that the increase in Ach sensitivity occurs at a time when there is an increase in rapid cell division. This possibility of receptor unmasking, however, has been eliminated by experimental evidence that strongly points to the synthesis of new receptors and their incorporation into the sarcolemma of the denervated muscle: (1) Upon denervation there is an increase in subsarcolemmal ribosomes which occurs concomittantly with an increase in Ach sensitivity (Gauthier and Dunn 1973; Gauthier and Schaeffer 1974). (2) The appearance of extrajunctional sensitivity can be prevented by inhibitors of protein synthesis (Gramp *et al.* 1972). (3) In freeze-fracture replicas the extrajunctional sarcolemma of denervated muscle shows the appearance of ~15nm intramembranous particles (on P-face) which are similar to

those in the junctional sarcolemma. These particles are either dispersed singly (Fig. 3) or aggregated in clusters (Fig. 4) (Tipnis and Malhotra 1976, 1977; Malhotra and Tipnis 1978). Such particles are not seen on the fractured faces of the non-synaptic region of innervated muscle, which indicates clearly that the particles of 15nm appear only after denervation. These particles are thought to be related to the AchR (Tipnis and Malhotra 1979a, b, c) and the aggregates correspond to the "hot spots" (Ko *et al.* 1977; Yee *et al.* 1978). Further evidence that the intramembranous particles represent AchR complexes comes from freeze-etch studies of ferritin- BGT labelled membranes from denervated muscle (Fig. 5) (Tipnis and Malhotra 1979a, b, c). (4) Incubation of muscle in a medium containing labelled amino acids results in the appearance of labelled receptors into the membrane (Brockes and Hall 1975b). Furthermore, new ^{125}I-BGT binding sites (Hartzell and Fambrough 1973; Devreotes and Fambrough 1976) and Ach sensitivity (Sakmann 1975) continued to appear even though the pre-existing receptors had been blocked with cold BGT. The appearance of these new receptor sites could be prevented by inhibitors of protein synthesis. From

autoradiography and kinetic studies it has been concluded that the AchR reside in the Golgi apparatus for 2-3 hours before they are incorporated into the sarcolemma (Hartzell and Fambrough 1973; Fambrough and Devreotes 1978).

Clusters of AchR

The extrajunctional receptors in embryonic and neonatal muscle and denervated muscle are not all uniformly distributed but may also occur as clusters (Sytkowski *et al.* 1973; Fischbach and Cohen 1973; Ko *et al.* 1977). The response to Ach in the region of clusters is considerably higher than elsewhere and therefore these clusters are often referred to as hot spots of Ach sensitivity (Ko *et al.* 1977). Freeze-fracture studies of identified hot spots in developing muscle show aggregates of 10-19nm intramembranous particles (Yee *et al.* 1978). The nonsynaptic sarcolemma of muscles denervated for 15 days has also been observed to have similar particles (15-18nm) which are distributed in a random manner (Fig. 3) or in clusters (Fig. 4) (Tipnis and Malhotra 1976; Malhotra and Tipnis 1978; Tipnis and Malhotra 1979a, b, c). Similar distribution of AchR has been observed by labelling the binding sites with ^{125}I-

BGT and studying their distribution in autoradiographs produced at light and electron microscope level (Tipnis and Malhotra, unpublished data). Such a distribution has been further substantiated by using ferritin- BGT in conjunction with freeze-etch studies (Tipnis and Malhotra 1979a, b, c). Prives *et al.* (1976) have noted in autoradiographs that the appearance of these clusters on myotubes of developing chick embryo seems to coincide with the appearance of cross-striations. As the cross-striations increase and are localized sharply within the myotubes, the clusters of AchR disappear from the surface. Such a transient occurrence of the clusters without neuronal influence has been suggested to reflect a sequential process during membrane differentiation (Prives *et al.* 1976).

The photobleaching technique used in the study of lateral motion of AchR in rat myotubes marked with fluorescent α-BGT indicates that the receptors that are uniformly distributed are mobile (diffusion constant $\sim 5 \times 10^{-11}$ cm^2/sec at 22°C), whereas those occurring in clusters are immobile. Selective photobleaching of the clusters shows that the receptors in the immobile and mobile phase do not exchange even after 10 hours in culture. The clusters themselves move slowly at a rate of 4μm/hr (Axelrod *et al.* 1976).

The mechanism of the stability or the formation of the Ach clusters is not understood except that the receptors in the cluster gradually disappear under electrical stimulation. Axelrod *et al.* (1978) have speculated that receptors in the clusters are stabilized by an immobile intra-or submembrane filamentous structure composed of molecules other than the AchR themselves. Microtubule and microfilament disrupting agents had no effect on the mobility of AchR, whether in a mobile state or in clusters. Braithwaite and Harris (1979) have reported that electrical activity of the nerve and/or muscle is required to suppress the appearance of extrajunctional receptor clusters and for the normal progress of muscle growth. These findings are based on experiments on embryonic rat muscle in which nerve and muscle activity were paralysed by the application of tetrodotoxin (TTX). In untreated embryonic rat muscle clusters of AchR (junctional) appear synchronously after 15 1/2 day gestation. TTX application at 16 day gestation did not alter the events of normal junctional AchR appearance but extrajunctional receptors appeared as they do in the denervated muscle.

The significance of the receptor aggregates is of current

interest. The suggestion that the clusters may be the sites of synapse formation has not been substantiated (see Frank 1979) by experiments on myocytes cultured from *Xenopus* larvae (Anderson *et al.* 1977). In these experiments, the receptors tagged with fluroescently labelled BGT showed the existence of patches under synapse that were different in shape from those existing in the extrajunctional region. During innervation the formation of junctional receptors takes place by aggregation of mobile receptors under the neurite while the adjacent patches disappear. It is conceivable that the receptor aggregates do not serve any particular role and are produced by a mechanism(s) akin to the formation of AchR in the postsynaptic membrane, i.e. during synapse formation a nerve could initiate AchR synthesis resulting in the formation of subneural clusters (Frank 1979). Factors other than nerves could also trigger cluster formation which can account for the appearance of clusters in muscle culture devoid of nerves (see Frank 1979). Also, Bevan and Steinbach (1977) have investigated the distribution of AchR in the developing skeletal muscle of rat by using autoradiography of ^{125}I- BGT. Their results are consistent with the data that the AchR are

mostly distributed uniformly on the myotubes, whereas they are essentially confined to the junctional region one week after birth. The loss of AchR from the extrajunctional region takes place gradually during development and the accumulation of the receptors at the junction takes place after the formation of the neuromuscular junction.

Comparison of Junctional and Extrajunctional Receptors

Although junctional and extrajunctional receptors both bind Con A, sediment at the value of 9S and have a similar reaction with serum raised against the eel AchR (Brockes and Hall 1975a), there are subtle differences in their pharmacological and biochemical characteristics. The extrajunctional receptors are less negatively charged (Brockes and Hall 1975a). Both the endplate potentials (epp) as well as Ach sensitivity are blocked by curare, although the concentration of curare required to block the Ach sensitivity in chronically denervated diaphragm and soleus muscles of rat is much higher than that necessary to block the epp (Lapa *et al.* 1974). The binding of ^{125}I- BGT to extrajunctional receptors is much less stable than that of junctional receptors. The innervated and denervated diaphragm and soleus muscles of mouse in which epp and Ach sensitivities are blocked by BGT show a recovery of response to Ach (50-75mv/nc) in the extrajunctional region of denervated muscles when the muscles are washed for 4-7 hours. In contrast, only a slight recovery of epp (0.5-1mv) was recorded in innervated muscle (Chiu *et al.* 1974). Almon and Appel (1976a) reported the existence of two BGT binding sites with affinity

constants of 10^9 M^{-1} and 10^5 M^{-1} in rat skeletal muscle. As a result of denervation there is a 28-fold increase in the high affinity site and a 5-fold increase in the low affinity site. The long term exposure of normal muscle to the non-ionic detergent Triton X-100 for 8-10 days results in the conversion of all binding to the high affinity site (10^9 M^{-1}). In a parallel experiment with denervated muscle there was no change in the binding affinity of AchR. Such differences in receptors from innervated and denervated muscles have been suggested to reflect different molecular interaction of the receptor with the environment, rather than a difference in the primary structure of the receptor protein (Almon *et al.* 1974).

Almon and Appel (1975) reported that γ globulin fraction from myasthenic patients binds to the high affinity site in denervated muscle but not to that present in innervated muscle. Further, Weinberg and Hall (1979) have reported that the extrajunctional receptors contain determinants that can be detected by sera from myasthenic patients. However, all of the determinants of the junctional receptors detected by myasthenic sera are present on the extrajunctional receptors so that myasthenic sera contain two classes of antibodies, one directed against determinants present

in both junctional as well as extrajunctional receptors and the other directed against determinants present only on the extrajunctional receptors. It is worth a note that antisera from several animals immunized with rat extrajunctional receptors, eel or *Torpedo* AchR could not reveal differences in the two classes of receptors. In this respect, myasthenic sera may provide a valuable means to distinguish the molecular species of AchR.

The junctional and extrajunctional receptors differ markedly with respect to their stability in the membrane. The stability of the toxin-receptor complex of rat diaphragm muscles studied *in vivo* (Berg and Hall 1975) or *in vitro* (Berg and Hall 1975; Devreotes and Fambrough 1975) indicates a rapid and continuous turnover of the extrajunctional receptor with a half-life of 14-18 hours. The junctional receptors have a much longer half-life, i.e. six days.

Merlie *et al.* (1979) have compared the characteristics of junctional and extrajunctional AchR in organ cultured adult rat diaphragms and have found that the degradation of the two types of receptors is similar in many ways, the point of difference being in the rate constant (10-fold difference) for the degradation process. The similarities lie in that the degradation of

both requires energy, both are inhibited by colchicine, both are inhibited by specific lysosomal protease inhibitors (leupeptin + pepstatin) and both are stimulated by anti-AchR antibodies. The difference in the rate of internalization of both receptors is speculated to be due to some structural difference in the membrane or the membrane-associated proteins.

The fluctuation analysis used for determining the size and duration of the elementary current indicates that the life-time of the Ach-induced channel is much shorter in the innervated muscle. The various values reported are 3.2 msec in frog (Colquhoun *et al.* 1975), 1 msec in rat (Sakmann 1978) and 1.54 msec in human muscles (Cull-Candy *et al.* 1978). The 4-5 times higher values are reported for extrajunctional channels in human myotubes (Bevan and Kullberg 1978) and in denervated muscles of frog (Neher and Sakmann 1976) and rat (Sakmann 1978). It appears that there may be species variation in respect of organization of receptors. For example, Schuetze *et al.* (1978) have reported that the extrajunctional and junctional AchR in chick myotubes are similar in respect to the mean channel open-time (3.0-3.5 msec at 24°C). Stability determined from autoradiography of cultures labelled with ^{125}I-- BGT indicates similar rates for junctional-

and extrajunctional-toxin receptors. These results are in agreement with those of Burden (1977a, b) who found a half-life of 30 hours for both junctional and extrajunctional receptors in embryonic posterior latissimus dorsi of chick. The rates of degradation undergo a change during development and junctional receptors acquire a half-life of five days after three weeks. These differences between the chick myotubes on the one hand and the rat and frog muscle on the other could be attributed to the different time period in the observations, since synapse formation may be a long-drawn process and changes may arise during subsequent development (Schuetze *et al*. 1978). The conductance of the junctional and extrajunctional channel in rat muscle is similar [34 ps($1s = 1\Omega^{-1}$] (Sakmann 1978). In frog sartorius muscle, however, conductance of the extrajunctional channel is 7.5 pmho as compared to 17.9 pmho in the junctional channel of innervated muscle (Dreyer *et al*. 1976). Such differences in conductance in the case of frog muscle indicate that the selectivity filter (portion of membrane channel which determines the ion selectivity of the channel) is smaller than in the extrajunctional channel. However, the permeability response to a wide range of organic cation in innervated and denervated frog sartorius muscle is similar, suggesting that the differences

in conductance must be attributed to some other factor and are not due to a difference in the selectivity filter of junctional and extrajunctional channels (Guy *et al.* 1977).

Although extrajunctional receptors from embryonic and denervated muscle have many common characteristics, they differ in their response towards curare. In intercostal muscles of rat embryo and neonatal rats, curare produced localized contractions and action potentials which are resistant to TTX but can be blocked by BGT, thereby suggesting that the action of curare is AchR specific. In parallel experiments with denervated muscle curare did not produce any depolarization. These results suggest either the existence of a third category of AchR in embryonic muscle or the difference in molecular environments in embryonic and denervated muscle (Ziskind and Dennis 1978).

Conclusions

It is apparent that the sarcolemma is a valuable model system for the investigation of the correlation of the structure and function of membranes. In the synaptic region it has two major proteins, AchE and AchR, and thus has a relatively simple composition as ccmpared to the membranous organelles of eukaryote cells. The sarcolemma is amenable to investigations by a variety of current techniques *in vivo*. There is an obvious compartmentalization of function along the sarcolemma, as manifested by the neuromuscular junction, which results from interactions as yet not understood between the nerve and muscle. Denervation leads to synthesis of the extrajunctional receptors which are incorporated into the sarcolemma but how these AchR complexes differ from the junctional receptors is not clear.

Betz and Changeux (1979) have reported that cyclic nucleotides are implicated in the regulatory control of the synthesis of extrajunctional receptors. Based upon experiments on cultured chick myoblasts, they proposed that dibutyryl cyclic GMP represses the synthesis of AchR and abolishes the development of Ach sensi-

tivity, whereas dibutyryl cyclic AMP increased the number of α-BGT binding sites. The repression of AchR synthesis by cyclic GMP may be a result of an increase in Ca^{2+} which enters the cell during the opening of Na^{+} channels by electrical activity. In frog sartorius muscle the extrajunctional receptors are subject to seasonal variation. They are absent in summer, appear in autumn and increase in winter. No such variation is reported to occur with respect to the junctional receptors. The observed variation in the extrajunctional receptors, therefore, has been suggested to be due to the variation in motor activity (Feltz and Mallart 1971).

The available data leads one to conclude that the junctional and extrajunctional receptors may differ in the biological role(s) they serve and it is an interesting question as to what these roles are and how they are controlled. There are also clusters of AchR in the developing muscle and in the extrajunctional sarcolemma of denervated muscle. How these clusters are formed, what role(s) they play and how they differ from the dispersed AchR are questions that are important in understanding nerve-muscle interactions. AchR manifest organizational alterations during development (Burden 1977a, b; Steinbach *et al*. 1979). It is likely that nonmembranous

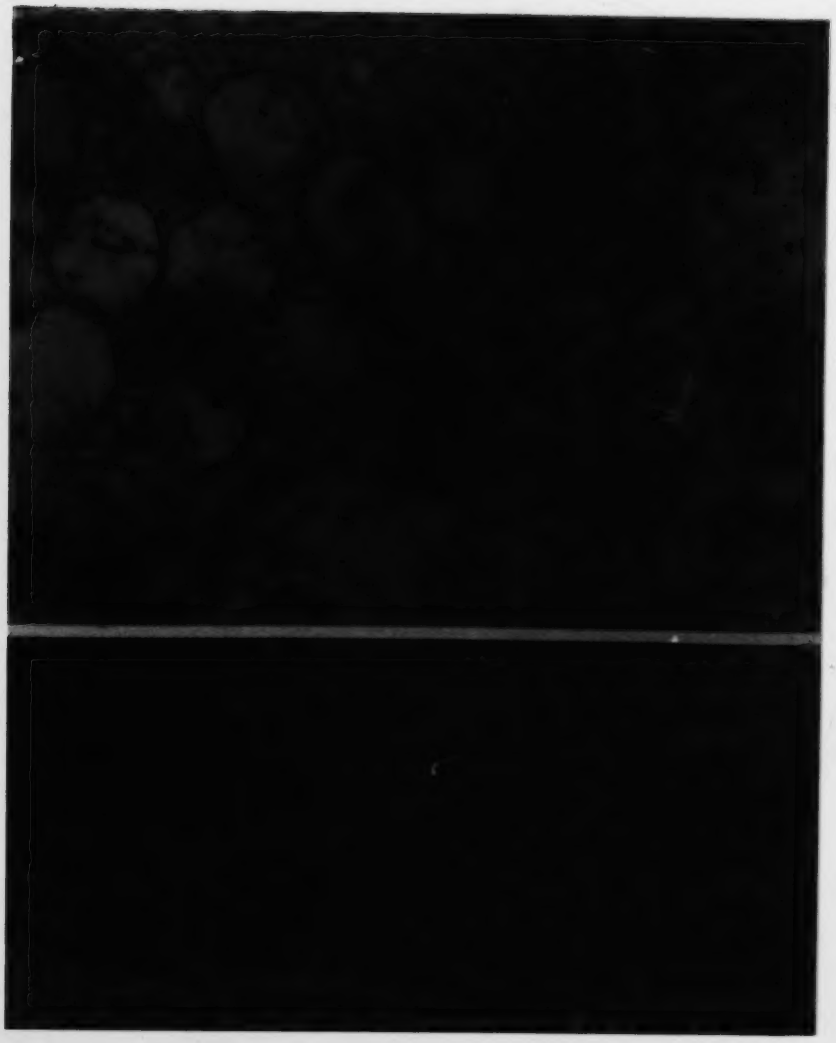
components, such as microfilaments and microtubules (Heuser and Salpeter 1979), play a role in the architectural organization of sarcolemma. They may exercise regulatory control on the mobility of AchR complexes within the sarcolemma. Antibodies influence the rate of degradation of AchR as evidenced by studies on myasthenic neuromuscular junctions (Drachman *et al.* 1978; Fambrough 1979) and on diaphragm of rats immunized with purified AchR (Heinmann *et al.* 1978). Electric field applied to embryonic muscle cell perturbs the AchR which then accumulates at one pole (Orida and Poo 1978). It is through the interplay of such diverse cell activities that the biology of AchR associated with the plasma membrane of muscle cells may be regulated.

Acknowledgements

The technical assistance provided by C. Saunders is greatly appreciated. The award of research grants (A 5021) by the NSERC is thankfully acknowledged. Mrs. D. Arbuthnott has taken great pains in handling the typing of this manuscript and the preparation of the references.

Figure 1. Electron microscope autoradiograph showing a neuromuscular junction of extensor digitorum longus (EDL) of rat. The muscles were incubated for 2 hr in oxygenated Kreb's ringer at 37°C containing ^{125}I- BGT (2µg/ml). After incubation, the muscles were washed thoroughly in several changes of ringer for 1-2 hr and processed for electron microscopy. The sections were coated with Ilford L_4 emulsion, developed in microdol x and fixed in sodium thiosulphate. The autoradiograph shows concentration of silver grains at the neuromuscular junction.

Figure 2. Electron microscope autoradiograph of denervated EDL of rat showing the presence of silver grains on the nonsynaptic sarcolemma. The experimental conditions and procedure for autoradiography were the same as given in Figure 1. It should be emphasized that comparable micrographs from innervated muscl- show a much lower density of silver grains in the nonsynaptic sarcolemma (Tipnis and Malhotra, unpublished).



Figures 3. & 4. Freeze-fracture electron micrographs of denervated EDL muscle showing the presence of 15nm (15-18nm) intramembranous particles on the P-face of non-synaptic sarcolemma, distributed randomly in Figure 3 and as clusters in Figure 4. It is emphasized that such particles are lacking from the comparable face of the innervated sarcolemma. The arrow indicates the direction of shadowing.

Figure 5. Freeze-etched membrane vesicle from denervated EDL muscle. The membrane vesicles were prepared and incubated in ferritin- BGT conjugate for 1 hr (details given in Tipnis and Malhotra, 1979c). The membranes were extensively washed, fixed in glutaraldehyde and frozen in nitrogen slush. They were fractured, etched (2 min) and replicated in Balzer high vacuum freeze-etch unit. The micrograph shows a vesicle which is labelled with ferritin at the surface (shown in double arrows). Running across the middle of the membrane vesicle appears to be a collagen fibre which indicates that the surface of the vesicle is exposed in the replica. Arrow in the right lower corner indicates direction of shadowing.

0.5um
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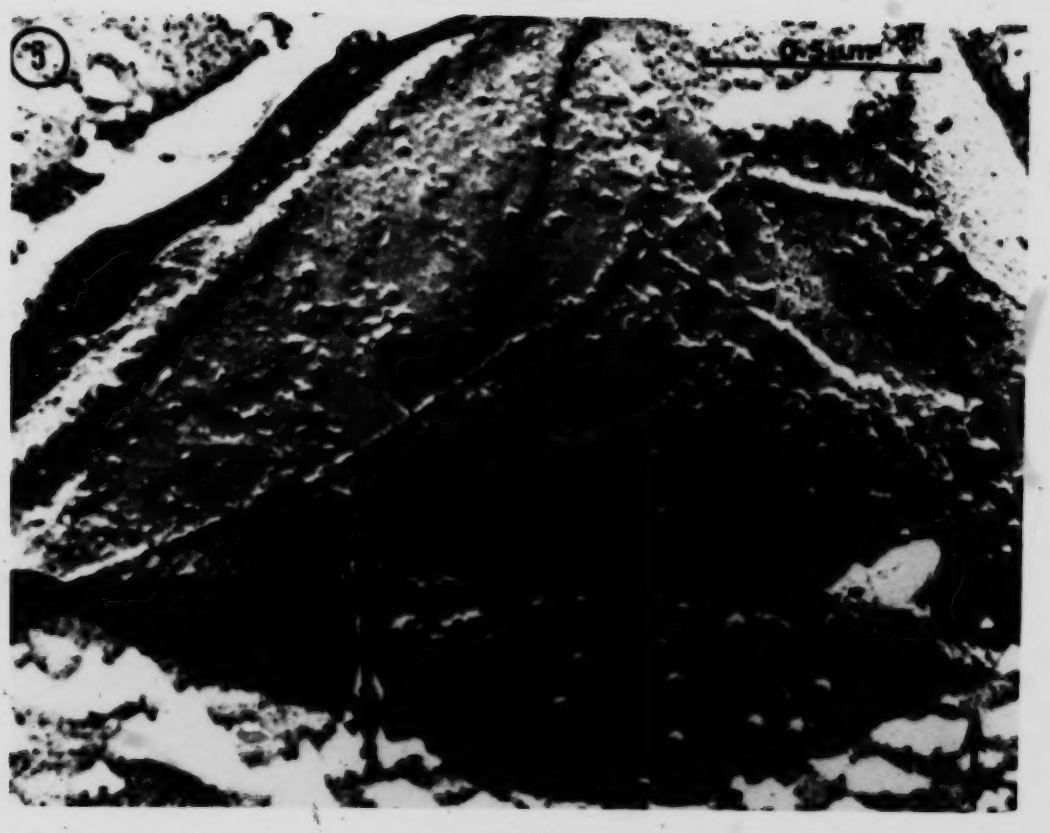
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CHAPTER VI

CONCLUSIONS

CONCLUSIONS

My investigations into the organization of acetylcholine receptors (AchR) and acetylcholinesterase (AchE) in denervated skeletal muscle of the rat have led to observations and conclusions, the most important of which are enumerated below (Fig. 1a, b).

Acetylcholinesterase (AchE)

(i) Histochemical reaction in the synaptic cleft of innervated and denervated muscle is specifically due to AchE.

(ii) Evidence is in favour of localisation of one particular molecular form of AchE, namely 16S (as against 4S and 10S). Denervation causes a decrease in AchE (16S) demonstrable by histochemical reaction at electron microscope level.

(iii) Intramembranous particles observed on the fractured face (P-face) of the synaptic sarcolemma are not obviously related to AchE molecules.

Acetylcholine Receptor (AchR)

(i) 15 nm intramembranous particles emerge on fractured face (P-face) of non-synaptic sarcolemma following denervation of skeletal muscle. This category of particles is lacking from the comparable fractures of the innervated muscle. Based upon the available physiological and biochemical data, it is known that the extrajunctional sensitivity of non-synaptic sarcolemma following denervation results from the synthesis of new AchR and their incorporation into the non-synaptic sarcolemma. 15 nm intramembranous particles were proposed

to be related to the AchR (Tipnis and Malhotra, 1976).

(ii) By using autoradiography (^{125}I-α-BGT) and freeze-etching of labelled membranous vesicles, evidence has been presented that the distribution of α-BGT binding sites in the non-synaptic sarcolemma in denervated muscle corresponds to the distribution of intramembranous particles. It is therefore concluded that the 15 nm intramembranous particles are the components of the AchR complex. There are two categories of AchR, singly dispersed and in clusters and there is similar distribution of 15 nm intramembranous particles.

(iii) AchR extends beyond the lipid bilayer and this has been demonstrated by using ferritin-α-bungarotoxin conjugate in conjunction with freeze-etching studies on membranous vesicles isolated from denervated muscle.

(iv) Based upon quantitative electron microscope autoradiography, a value of approximately 14,000 binding sites/μm^2 relative to the synaptic sarcolemma has been obtained. In contrast, there are ~25 α-BGT binding sites/μm^2 in the non-synaptic sarcolemma in innervated muscle. The non-synaptic sarcolemma of the denervated muscle has 1,590 sites/μm^2. These estimates are based upon the assumption that there is one binding site per AchR. These values for the α-BGT binding sites in the synaptic sarcolemma of the innervated muscle and non-synaptic sarcolemma in the denervated muscle are 3-5 times higher than the estimated density of intramembranous particles. It would therefore appear that each intramembranous particle contains either (i) two or more binding sites and/or (ii) is composed of receptor units.

Figure 1. Diagramatic representation of the comparison of the structure of sarcolemma in innervated (Fig. A) and denervated (Fig. B) skeletal muscle. Note the appearance of AchR in the non synaptic sarcolemma and their increase (*) in the subsrcolemmal sarcoplasm (SS) in the denervated muscle and their virtual absence in the corresponding locations in the innervated muscle. Also note the appearance of large intramembranous particles on the P face of the non synaptic sarcolemma (PNS) in denervated muscle and their absence from the corresponding face in the innervated muscle.

AchE, acetylcholinesterase; AchR, acetycholine receptors identified as α-bungarotoxin binding sites (*); ES, PS, ENS and PNS, are the explasmic and plasmic fractured faces of the synaptic and non synaptic sarcolemma. MP, membrane protein; N, Nucleus; NT, nerve terminal. Not to scale.

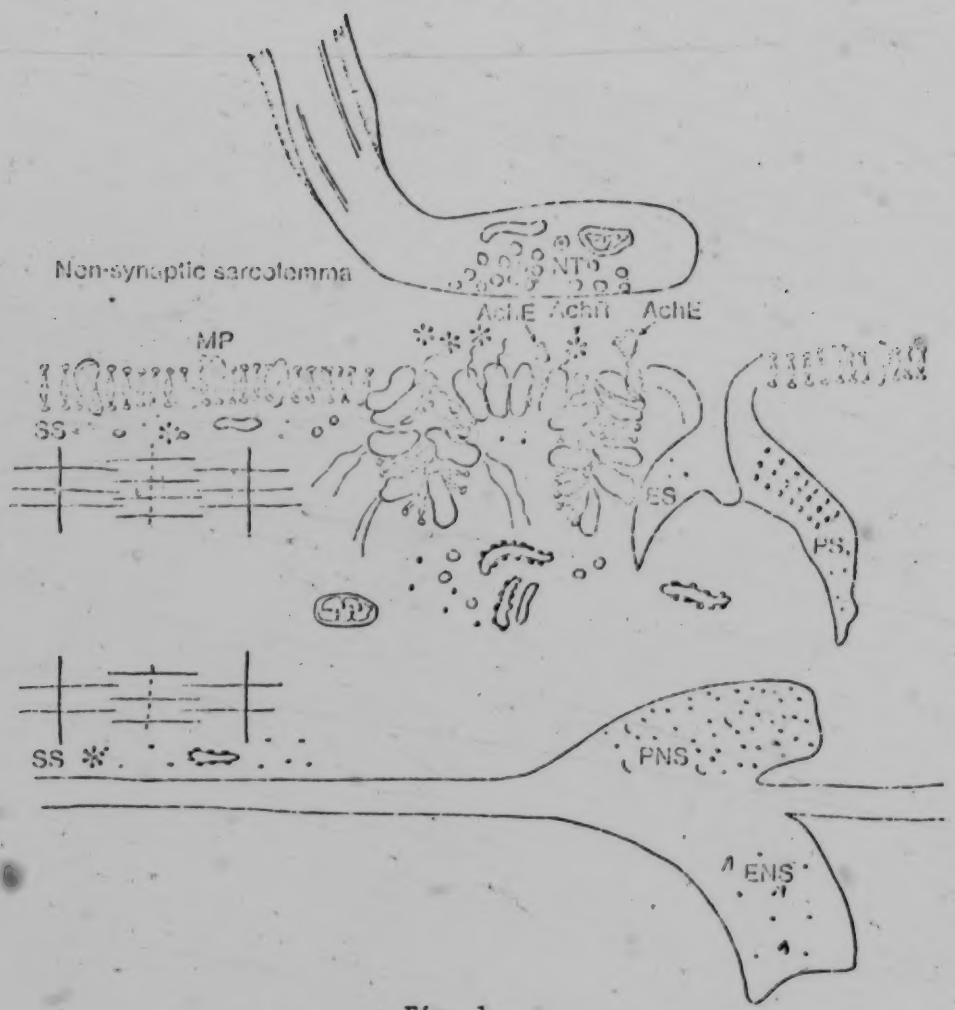
Non-synaptic sarcolemma
NT
AchE
AchT
AchE
MP
SS
ES
PS
SS
PNS
ENS

Fig. 1a

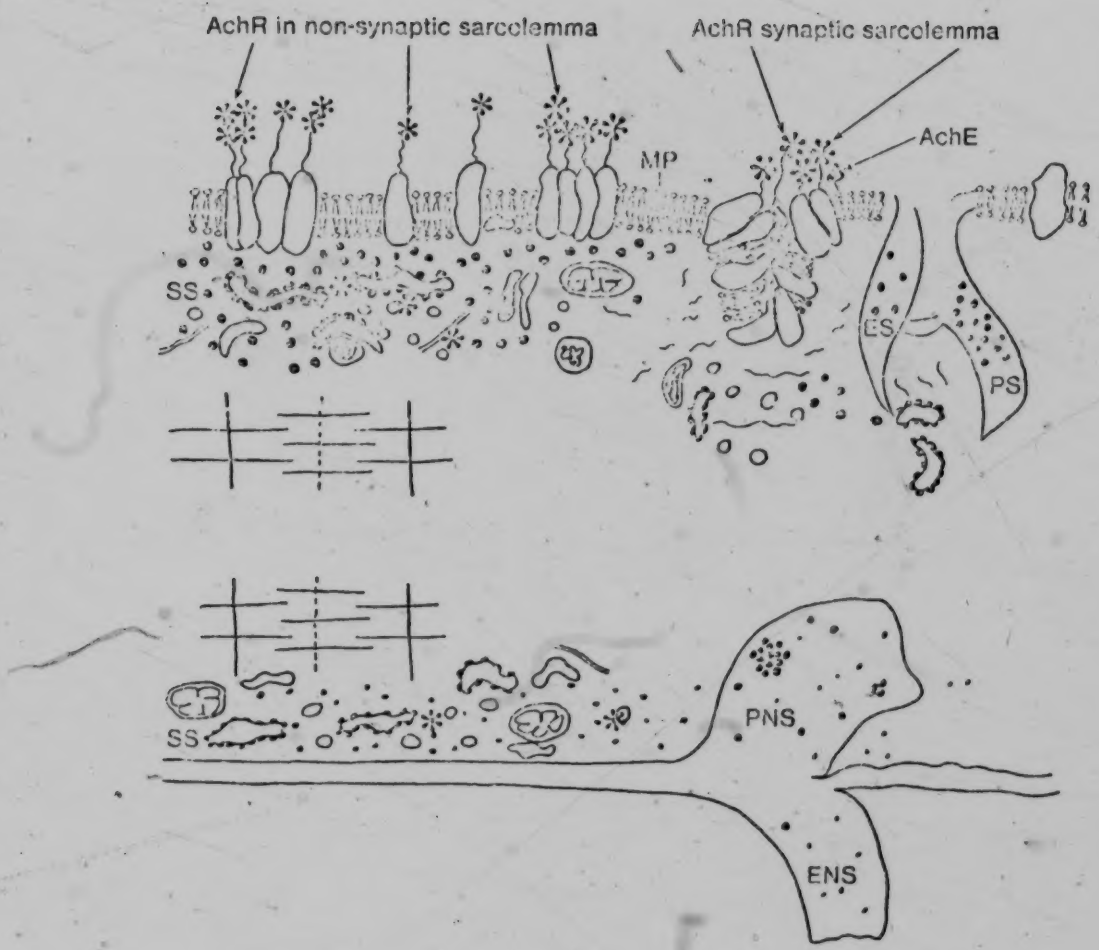
AchR in non-synaptic sarcolemma
AchR synaptic sarcolemma
AchE
MP
SS
ES
PS
SS
PNS
ENS

Fig. 1b

APPENDICES

APPENDIX I

Preparation of Crude Sarcolemmal Membrane

In the present investigation, the sarcolemmal membranes were prepared as crude membrane fragments by the procedure of Boegman et al. (1970) as well as by the evacuation procedure according to Beringer and Koenig (1975). In several experiments, the crude membrane fraction proved suitable for localisation of Ach receptor in thin sections but not for freeze-fracturing studies. Therefore, evacuated membranes were prepared by the method of Beringer and Koenig (1975). The results on visualisation of Ach receptor are based on both methods described below.

(1) Preparation of Crude Membrane Fragments (Boegman et al., 1970)

The animals were sacrificed by cervical fracture and muscles of the lower leg quickly transferred to sucrose medium (0.25 M sucrose, 0.2 mM Na_2EDTA, 0.1 M Tris buffer (pH 7.6)) at 0° C. The muscles were cut into minute segments with microscissors and connective tissue, blood vessels and large nerve fibers were removed.

The muscle pieces were homogenized in sucrose medium in a vortex homogenizer for 1½ minutes at a setting of 60 and then for a minute at a setting of 40 after dilution with sucrose medium. The homogenate was filtered through cheese cloth and centrifuged at 900 x g for 7 min. The pellet was suspended in equal volume of cold water and to this a volume of 0.8 M LiBr in 0.02 M Tris buffer (pH 8.5) equal to the volume of the water was added. The suspension was slowly stirred for 10-12 hr at 0° C and then centrifuged at 1,000 x g for 5 min. The pellet from

this centrifugation was discarded and the supernatant centrifuged at 105,000 x g for 30 min. The pellet was suspended in 0.6 M KCl in 0.01 M Tris buffer (pH 8.0) and centrifuged at 105,000 x g for 30 min. The pellet was again suspended and rehomogenized in the KCl-Tris medium. The next centrifugation was carried out at 7,000 x g for 15 min and the supernatant was recentrifuged at 105,000 x g for 30 min. The pellet was washed twice with cold double distilled water by centrifugation at 105,000 x g for 30 min, and the pellet obtained provided the source of membrane used in this investigation. The protein concentration was measured by Lowry's method (1951) and the preparation was divided into a number of aliquots and stored at -20° C.

(2) Preparation of Muscle Membrane by Evacuation Procedure (Beringer and Koenig, 1975)

The muscles after excision were rinsed in 0.15 M NaCl and cut longitudinally into fine segments with a pair of scissors. The segments of muscle were transferred to 1-2 mM $CaCl_2$ and 0.3 mM Tris HCl buffer (~4° C; pH 7.8-8.0) for 10-15 min, and incubated further in the same but fresh buffer for 5 hr at 4° C. The muscles were then rinsed for 10-15 min in 0.3 mM Tris HCl buffer (pH 7.8-8.0) containing 30 mM KCl or NaCl, and incubated in this buffer for 5-12 hr at 4° C. The muscle fibers were then rinsed in double distilled water for 5-10 min and were cut into segments approximately 4-5 mm in length. The evacuated muscle fibers were pipetted into fresh distilled water for further evacuation.

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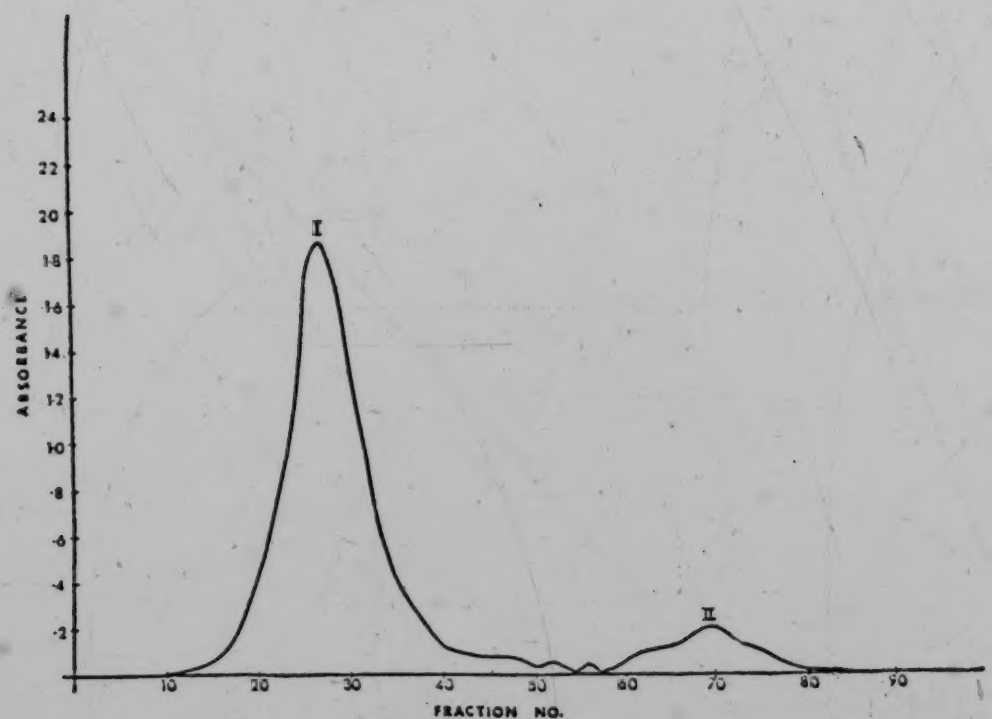
APPENDIX II

Preparation of Ferritin-α-Bungarotoxin (Ft-α-BGT)

α-bungarotoxin (α-BGT) supplied by Miami Serpentarium, Florida, was used for the above conjugate. Ferritin (6 x crystallised, cadmium free) obtained from Polysciences was conjugated to α-BGT by using glutaraldehyde according to the method of Hourani et al. (1974). The incubation mixture contained 675 µl distilled water, 100 µl 0.05 M NaH_2PO_4, pH 7.2, 125 µl ferritin (92 mg/ml), 40 µl α-BGT (0.5 mg). This mixture was stirred and to it 50 µl of 1% glutaraldehyde was added, 5 µl each time over a period of 10-15 min. The above mixture was incubated for 2 hr at room temperature and 0.5 M NaCl was added. The mixture was applied to Sephadex G-200 column. The columm was equilibrated with .05 M NaH_2PO_4 (pH 7.2) and 0.5 M NaCl. Before application of incubated mixture, albumin was passed through the column to saturate the nonspecific sites in the column facilitating the efficient separation of bound and unbound α-BGT. The buffer was run through the column after ½ hr and then incubated mixture of ferritin-α-BGT was applied. The column was eluted with buffer and 0.1 ml aliquots of fractions were collected. Protein concentration of these aliquots was determined by Lowry's method. The first peak was collected (Fig. 1) and dialysed overnight against Earl's balanced salt solution which contained 0.025% sodium azide. The dialysate was separated in different vials and stored at -20° C until used.

FIGURE 1. Separation of Ft-α-BGT conjugate on Sephadex G-200. Sephadex G-200 was equilibrated in 0.05 M NaH_2PO_4 and 0.5 M NaCl. The incubated mixture of ferritin, α-BGT and glutaraldehyde was passed through the column and eluted with the above buffer. The first peak is of Ft-α-BGT conjugate and the second peak is probably the unconjugated α-BGT.


ABSORBANCE
24
22
20
18
16
14
12
10
8
6
4
2
I
II
10
20
30
40
50
60
70
80
90
FRACTION NO.

REFERENCES

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APPENDIX III

Incubation of Membranes in ^{125}I-α-BGT

The membranes were assayed for their Ach receptor activity by using the filter-assay procedure suggested by Klett et al. (1973); 1 ml of Kreb's ringer contained 50 µg of membrane protein, ^{125}I-α-BGT (2×10^{-9} M), 1% (w/v) Tween 80. Control samples were incubated with α-BGT (10^{-4} M) for 1 hour prior to incubation with ^{125}I-α-BGT. In experiments on time course binding, innervated and denervated membrane preparations were incubated with ^{125}I-α-BGT for varying periods of time. To determine specific binding for membrane preparations of denervated muscle, several aliquots containing equal amounts of membrane protein but varying amounts of ^{125}I-α-BGT were incubated. In this experiment, the specific binding was studied by first incubating the samples with cold α-BGT for 1 hr and then with labelled toxin. The samples after incubation were immediately filtered through DEAE cellulose (81). After filtration each sample was rinsed with 50 ml of buffer after which the filter discs were dried and counted in a gamma counter.

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APPENDIX IV

Quantitative Analysis of Autoradiographs

I. The localization of radioactive source was done by the method of Fertuck and Salpeter (1976). The sections from the endplate region were randomly chosen; they were photographed and printed at a magnification of 30,000X. For determination of the distribution of radioactivity, all grains within 30 mm (on micrographs) on muscle as well as on axonal side were considered. The smallest circle circumscribing the grain was marked over the grain and its center was punched. The perpendicular distance from the grain center to the postjunctional membrane was measured. A histogram of grain distribution versus distance was plotted for both axonal as well as muscle side. A transparency made from graph paper (with distance between intersections = 2 mm) was placed over the circle and all grid intersections within the circle were punched. The perpendicular distance from each grid intersection to the postjunctional membrane was measured. The histogram of lattice points was plotted. Dividing the total number of grains in each column by the lattice points in the same column gives the grain density/unit area.

For determination of the HD (half-distance) value for this investigation, the grains that were considered above were added for each unit distance from the source and the integrated curve of grain distribution was plotted against the distance (Fig. 1). The number of grains rises linearly only up to certain distance after which it levels off. Half-distance in autoradiography is defined as the distance containing 50% of the total grains. From the integrated distribution curve

in this study the HD value of 100 nm was determined.

The grain density was normalized by setting the value at the source to 1 and after adjusting other values proportionately was plotted against distance in HD units. The histogram drawn from these values indicates (i) as to whether or not the curve resembles the theoretical curve given for line source by Salpeter et al. (1969), and (ii) the degree of radiation spread from the source.

II. Estimation of α-bungarotoxin sites/μm^2. The α-bungarotoxin (α-BGT) binding sites/μm^2 were determined from the following formula given by Fertuck and Salpeter (1976).

$$\text{Sites}/\mu m^2 = \frac{G \times d}{124800\ (1 - e^{-0.01155t})} \times \frac{A}{Soc}$$

where G = grains/μm^2 of membrane area; d = inverse of sensitivity (i.e., number of decays required to produce one grain; A = exposure time in days; So = specific activity of the ^{125}I-α-BGT at the beginning of exposure period (Curies/millimole); c = disintegrations/min or 2.22×10^{12}; A = Avogadro's number in same unit as for So (e.g., 6.023×10^{20} molecules/millimole).

The grain density (grains/μm^2) and the sensitivity required for the estimation of binding sites were determined as follows:

(a) Determination of membrane area and grain density. Membrane area was determined according to the procedure given by Fertuck and Salpeter (1976). The length was measured by counting the number of times the lines from a two-dimensional grid placed over the autoradiogram

intersected the membrane and calculated from the following formula:

$$L = d \times a/2 \times \pi/2$$

L = length of the membrane

d = spacing of the grid in micrometers at the magnification of electron micrograph

a = number of intersections

Area is then determined by multiplying the length by the thickness of the section (~100 nm). All grains within 4 HD values were counted and the grain density/μm^2 relative to sarcolemmal membranes were calculated.

(b) Determination of sensitivity. Sensitivity is the inverse of the number of nuclear decays to give on the average one developed grain. In this investigation, the sensitivity was calculated according to the procedure given by Williams (1977), and is given below.

The specific activity of ^{125}I-α-BGT = 230 μci/ml = .23016 millicuries/ml.

1 millicurie = $2.22 \times 10^6 \times 1000$ disintegrations/minute.

Therefore .23016 millicurie will have $.23016 \times 2.22 \times 10^6 \times 1000$ disintegrations/minute (DPM).

Volume of ultrathin section = .5 mm x .5 mm x 100 nm = 1.25×10^{-9} ml.

DPM/ultrathin section = $.23016 \times 2.22 \times 10^6 \times 1000 \times 1.25 \times 10^{-9}$.

In 35 days there will be 35 x 24 x 60 minutes.

DPM/ultrathin section in 35 days

$= 35 \times 24 \times 60 \times .23016 \times 2.22 \times 10^6 \times 1000 \times 1.25 \times 10^{-9}$.

Area of ultrathin sections = .5 x .5 x mm^2 or 2.5×10^6 μm^2.

DPM/ultrathin section in 35 days/μm^2

$$= \frac{35 \times 24 \times 60 \times .23016 \times 2.22 \times 10^6 \times 1000 \times 1.25 \times 10^{-9}}{2.5 \times 10^6}$$

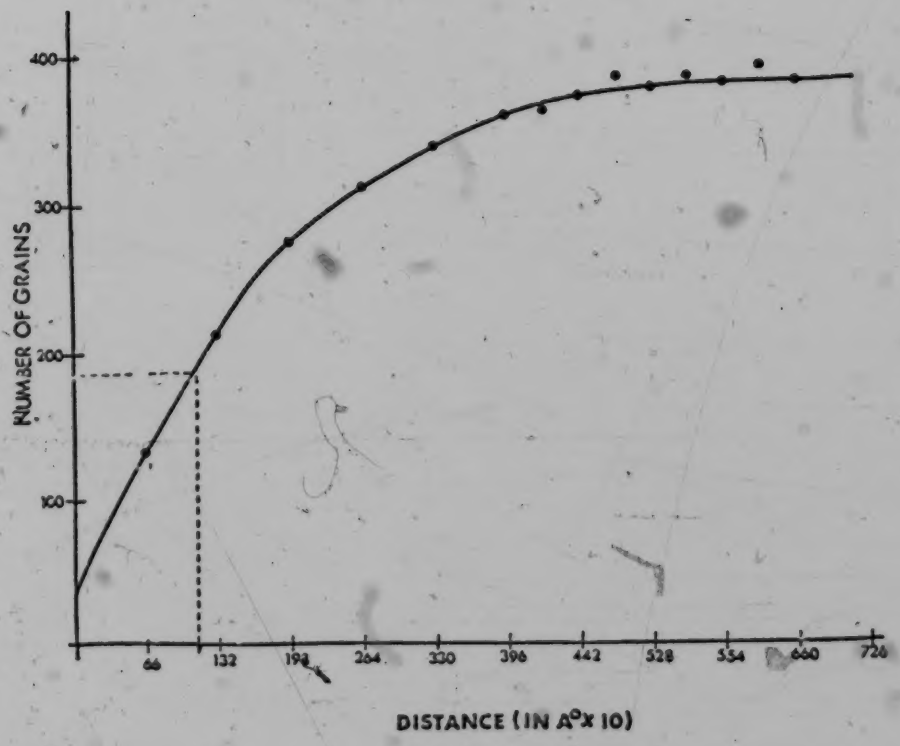
= .0128 disintegrations in 35 days/μm^2.

The density of grains $2.4/\mu m^2$

$$\text{decays required to produce one grain} = \frac{.0128}{2.4} = .005365.$$

$$d = \frac{1}{\text{decays required to produce one grain}} = \frac{1}{.005365} = 186.39.$$

FIGURE 1. Integrated grain distribution of silver grains in neuromuscular junction labelled with ^{125}I-α-BGT. The pale gold sections (100 nm) are coated with Ilford L_4 emulsion and developed in microdol X. The grains are counted from the source (the line drawn at the top of synaptic sarcolemma) and are added consecutively with increasing distance from the source. The graph shows the total number of grains from the source. The distance that includes half of the total grains is the half-distance (HD) and is 100 nm in the present study.


400
300
200
100
NUMBER OF GRAINS
66
132
198
264
330
396
442
528
554
660
726
DISTANCE (IN A°x 10)

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